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Health Effects of Nitrates in Water

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The results of the epidemiological studies indicate that infants consuming appreciable amounts of water high in nitrates in the form of powdered milk formula show significantly raised methemoglobin levels. This is also true for infants consuming tap water having a nitrate concentration ranging from 45-55 milligrams per liter.

Laboratory studies of acute and chronic exposure used nitrites and found indication that nitrites can pass the rats placenta and cause raised methemoglobin levels in the fetus; that pregnant rats are particularly sensitive to exposure to nitrites, that pups born to dams exposed to nitrites during gestation show poor growth and development; and that rats exposed to sodium nitrate as well as sodium nitrite in their drinking water for 18 months show distinct deviations in heart blood vessels even at the level of 200 mg/liter of $NaNO_2$. Behavioral effects were noted in mice exposed to high concentrations of nitrite in drinking water.

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HEALTH EFFECTS OF NITRATES IN WATER

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FOREWORD

The United States drinking water standards listed a recommended limit for nitrate in 1962 because of the occurrence of cases of Methemoglobinemia in infants that consumed water with a high nitrate concentration. But many infants were healthy even when their water supply was high in nitrate and more information was needed to support a specific limit for nitrate in drinking water.

An opportunity to expand research on the health effects of nitrate occurred when counterpart funds were available in Israel where it was known that many water supplies had high nitrate concentrations. Another balanced research effort has been carried out comprising the development of clinical chemistry techniques, toxicological studies, and epidemiological surveys.

The first epidemiological survey demonstrated that nitrate in drinking water did not pose a public health problem because the water was not consumed by the infants, but subsequently a study population was obtained in the Gaza strip where the infants were exposed to the water with an excessive nitrate concentration.

The nitrite concentration of drinking water is low or nonexistant but this ion was used in most of the toxicology studies because the animal model did not allow for the nitrate-to-nitrite conversion in the gut. Thus the findings may be more applicable to the food preservation problem than to drinking water.

Many interesting findings suggest further research.

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ABSTRACT

This report represents the results of a series of field and laboratory studies designed to evaluate the health effects of nitrates in drinking water.

The results of the epidemiological studies indicate that infants consuming appreciable amounts of water high in nitrates in the form of powdered milk formula show significantly raised Methaemoglobin levels. This is also true for infants consuming tap water having a nitrate concentration ranging from 45-55 ppm nitrate. It is felt that this latter finding provides direct epidemiological evidence in support of the current nitrate standard in drinking water of 45 ppm. Other field studies showed that even breast-fed infants or those receiving pasteurized milk can consume under Israel conditions considerable amounts of tap water as liquid supplements during the hottest months of the year.

Laboratory studies on the acute and chronic toxic effects of nitrites indicate among others, that nitrites can pass the rat's placenta and cause raised Methaemoglobin levels in the fetus; that pregnant rats are particularly sensitive to exposure to nitrites, and that pups born to dams exposed to nitrites during gestation show poor growth and development; that rats chronically exposed to sodium nitrate and sodium nitrite in their drinking water for 18 months show distinct deviations in heart blood vessels even at the level of 200 ppm of $NaNO_3$. Exposure of mice to nitrites in drinking water causes behavioral effects such as lowered motor activity and an increase in isolation induced aggression. A number of sensitive analytical micro-methods required for these studies were developed.

The results of the epidemiological and the toxicological studies provide little basis for a liberalization of the current drinking water standard for nitrates. If anything, evidence is presented which may raise some questions as to whether the current standard provides a sufficient margin of safety below the detectable effect level.

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SECTION I

CONCLUSIONS

1. The analytical methods developed in this project provide sensitive and reliable micromethods for epidemiological studies in which methemoglobin, nitrites and ascorbic acid levels in blood must be determined from samples taken in the field and brought to the laboratory for later analysis.

2. Evidence is provided that the ion selective electrode method developed to determine nitrate concentrations in water can lead to considerable inaccuracies when testing highly mineralized water. Chlorides can, in particular, lead to inaccurate results. The ion selective electrode method can at best be considered a screening test under field conditions and then only for water with nitrate concentrations over 45 ppm (as NO_3).

3. The results of the two epidemiological studies which encompassed 2891 infants up to 24 months of age indicate that there is a relationship between concentration of nitrates in drinking water consumed mainly as powdered milk formula and raised methemoglobin (MetHb) levels. The effect was detectable and significant even in the group of infants exposed to water containing 45-55 ppm of NO_3. Even though no clinical cases of methemoglobinemia were detected, it is felt that the appearance of a significant increase above the normal MetHb levels in infants when exposed to water with nitrate concentrations slightly above the current standard of 45 ppm is sufficient to provide direct epidemiological support for the current standard. The health significance of such subclinical levels of MetHb is unclear and it is still unknown why only a small number of infants exposed to such levels of nitrates in water develop clinical cases of the disease.

4. Methemoglobin is higher in the first three months of life and among infants with gastrointestinal disturbances irrespective of nitrate intake.

5. The results of the carefully controlled study of infants consuming nitrates in their formula in a hospital ward provided results very similar to those of the field studies. However, some evidence indicating an adaptive mechanism was obtained. This is not yet understood.

6. A study of liquid intake among 104 infants age 1-5 months indicated that while during the cool months 90% of the total liquid intake is made up of milk, as much as 50% can be in the form of tap water supplements during the hottest month. This finding may lead to considerable intake of nitrates from tap water even among infants not receiving tap water as part of their

powdered milk formula. This finding is in apparent conflict with the findings that even in high nitrate areas infants receiving no powdered milk formula in their diet had no higher levels of MetHb than those in low nitrate areas. No seasonal pattern of MetHb levels was determined. The specific role that powdered milk formula possibly plays in the development of raised MetHb in high nitrate areas still remains a moot point.

7. The low level of MetHb reductase (M.R.) in infants at birth and for the first few months of life may provide a partial explanation of the particular susceptibility of this age group. The role that partial M.R. deficiencies of a genetic form play in the development of clinical disease when exposed to nitrates in water is still an open question worth exploring.

8. The passage of nitrites from pregnant rats to the fetus through the placenta with the development of raised MetHb in the fetus opens the question as to whether only infants 0 to six months of age should be provided with low nitrate water in areas where the nitrate concentrations are high in the general water supply? This phenomenon was detected even at very low nitrite doses to the pregnant rat, (7.5 mg/kg of sodium nitrite). That is not much above doses that could occur under not too extreme environmental conditions. The potential significance of this finding to humans is emphasized by the fact that the MetHb reductase levels in the human fetus is only one-tenth of that found in newborn rats.

9. The higher sensitivity of pregnant rats to nitrites and the poor growth and development of pups born to dams exposed to nitrites during gestation suggests that a careful evaluation of the health effects of exposure to nitrites and nitrates during pregnancy is required.

10. Ascorbic acid was demonstrated to provide partial protection against methemoglobinemia in rats. The mode of action of ascorbic acid is still unclear.

11. The studies involving chronic exposure of rats to various concentrations of sodium nitrate and nitrite in drinking water indicate that even at very high dose levels few gross effects are detectable. However, minor deviations in heart blood vessels thickness were discernible after 12 months and became clear cut after 18 months of exposure to both nitrates and nitrites. The thin balloon-like blood vessels not typical in rats of this age were clearly detectable even at a dose of 300 mg/l of sodium nitrite. The pathological significance for humans of this finding is not clear. It appears that this effect may be due to direct toxicity of the nitrites rather than as a side effect of methemoglobinemia since in the group of rats exposed to nitrates no raised MetHb was detectable.

12. Evidence of an adaptive mechanism to nitrite exposure was detected in the chronic studies. DPG levels increase within 2 days of exposure to nitrites and remain elevated throughout the period of exposure. The effect of this is to increase the oxygen release from blood to the tissues.

15. The behavioral studies also indicate the possibility that nitrites have a direct toxic effect since the lowered levels of motor activity in mice and increased isolation-induced aggression occurred even in mice showing only very slight increases in MetHb. While the reduced motor activity might be associated with the known quality of nitrites as muscle relaxants, the increased aggression suggests a more central effect. While the studies on the effects of nitrites on brain electrical activity of rats remain inconclusive, the recent findings of Russian researchers in this area suggests that there is more to this matter than we have been able to clearly confirm.

16. Finally, it must be pointed out that from both the field and laboratory studies evidence has been gathered that nitrates and nitrites may be more toxic than generally considered. The fact that significant effect can be detected in infants consuming water having only slightly more nitrates than the current standard raises the question as to the margin of safety provided by that standard. The other toxic effects revealed in this study which may be independent of the methemoglobin increases resulting from exposure to nitrates raises many questions as to the proper basis for establishing the nitrate standard in drinking water which has until now been based on the possibility of infant methemoglobinemia alone. Certainly in light of this information there can be little grounds for relaxing the current standard, particularly in those areas where infants consume considerable amounts of tap water in the form of milk powder formula.

SECTION 16

RECOMMENDATIONS

While the nitrate standard for drinking water has been based originally on the association with infant methemoglobinemia, a number of findings in this study point to possible direct toxic effects not previously considered. The full significance of the pathology in heart blood vessels in rats exposed to nitrates and nitrites for 18 months should be thoroughly investigated both in the laboratory and in the field. The behavioral changes in mice such as reduced motor activity and increased isolation induced-aggression, also not directly associated with raised levels of MetHb, should be studied further. A complete rechecking of the findings of changes in the H.S.G. in rats exposed to nitrites in water should be urgently made.

The high degree of sensitivity of pregnant rats to methemoglobin inducing agents should be evaluated, since to date only infants up to 6 months in age have been considered at risk. The question of transplacental passage of nitrites and raised MetHb in the fetus of rats requires further evaluation as to its significance to humans.

There is still much to be learned about the association of nitrates in drinking water and raised MetHb levels in infants. What role does the powdered milk formula actually play in the development of MetHb? How effective are vitamin C rich foods such as citrus juice and tomato juice in preventing raised MetHb? What is their mode of action? Why do only a small percent of infants exposed to high nitrates in water develop clinical cases of methemoglobinemia? A fuller understanding of these questions is still required.

However, despite the many questions that remain unanswered it is apparent that nitrates and nitrites are potentially more toxic than generally assumed. The information gained from this project provides a basis for recommending that no relaxation be made in the nitrate standard for drinking water at this time despite the infrequency of clinical cases of the disease. Other direct toxic effects of nitrates may prove to be of equal or greater importance than the problem of infant methemoglobinemia. Until those questions are fully elucidated the standard would best be maintained as is while being kept under constant scrutiny and review.

SECTION III

INTRODUCTION

GENERAL

The standard for nitrates in drinking water was initially established based on limited epidemiological evidence that indicated that no cases of infant methemoglobinemia occurred in areas with less than 45 ppm of nitrates (as NO_3) in the water. Since the standard was established there has been considerable controversy on the subject. Some European researchers have reported that they were able to detect raised methemoglobin levels in so-called normal infants in areas where occasional clinical cases of the disease were reported. In addition, clinical disease was also reported among infants exposed to water having less than 45 ppm of nitrates. Suggestions that a stricter standard be enforced have been made as a result of such studies.

On the other hand, extensive areas in the United States supplying water showing nitrate levels above the standard have reported little or no clinical cases of the disease. Since nitrates are difficult to remove from water by economically feasible means, moves have been made for a more liberal standard reflecting the lack of clinical disease in such areas.

Since the epidemiological and toxicological base for establishing the nitrate standard was relatively limited the project reported upon here was initiated to clarify some of the basic questions concerned with the standard and provide a basis for confirming it or changing it as need be. What follows is a review of some of the general aspects and known pathogenesis of methemoglobinemia which served as the basic consideration for establishing the standard initially.

GENERAL ASPECTS OF METHEMOGLOBINEMIA

Methemoglobinemia can be caused by several chemicals such as nitrates, perchlorates, aminophenols, aniline, sulfonamides and others(1,2). Various endogenous forms of the disease are known. Nitrates do not directly convert hemoglobin to methemoglobin but can be converted to nitrites by intestinal microflora, with subsequent formation of methemoglobin. Hemoglobin (Hb) is the oxygen carrier of the blood. It is a protein of molecular weight 68,000, consisting of four identical sub-units, each containing a polypeptide chain (globin) and a heme group. Every heme contains at its center an atom of iron which, in the oxygenated form of Hb, oxyhemoglobin (HbO$_2$), is in the bivalent state (Fe^{2+} ferrous). Methemoglobin (MetHb) is the oxidized product of Hb in which the iron is in the trivalent state (ferric Fe^{3+}).

On transition from the ferrous to the ferric state the Hb loses its ability to combine with O_2. The exact molecular mechanism of the conversion of Hb to MetHb is still obscure.

Conversion of Hb to MetHb takes place all the time in the body, but the quantity of the latter is maintained at a low, steady-state level mainly by the action of an enzymatic system(3). Several enzymes with MetHb reduction capability have been purified, but the exact mechanism by which they operate *in vivo* has not yet been resolved. A direct, nonenzymatic reduction of methemoglobin is carried out by glutathion or ascorbic acid(4). The normal concentration of methemoglobin is still a matter of dispute.

Methemoglobinemia constitutes a potential impairment of the proper supply of oxygen to the tissues. For example, it was found that in trained subjects who underwent work tests, 10-12% MetHb resulted in impaired oxygenation of muscles(5). This phenomenon is a result of both less hemoglobin and the greater affinity of the residual Hb for oxygen.

PATHOGENESIS OF METHEMOGLOBINEMIA

It is accepted by many workers that under normal circumstances that about 1% of the total hemoglobin exists as MetHb(6,7). No external signs or symptoms are generally noted under 5%. The first signs of cyanosis can be seen between 5 and 10%(8).

The presence of high concentrations of nitrates in water is the principal determinant of the occurrence of methemoglobinemia in infants; however, it is not the only one(9). Other factors important in the pathogenesis of the disease are:

Age: Most of the cases of nitrate methemoglobinemia occur in infants below one year of age. In a review of 146 cases of methemoglobinemia in Minnesota, 90% of them were found to have occurred by the age of eight weeks. The youngest case was seven days and the oldest five months(10). Sandidt and Knotek(11) reported on a survey carried out in Czechoslovakia, in which 52% of the infants, 0-3 months old, in a high-nitrate area had elevated methemoglobin levels; in the age group from 3-12 months old the percentage reached 13. There are also reports

on elevated MetHb levels in older children and adults who had consumed water with high nitrate concentrations but showed no symptoms of the disease(12). In a Russian study(13), of 200 children in Ivy Murzelier, it was found that 97.3% of those who ingested water which contained 20-40 mg NO_3/l had elevated MetHb levels. In 50% of the cases the level was higher than 5%. Previously it was found that 5 mg/l nitrate did not raise the methemoglobin level(15).

Presence of bacteria: Cornblath and Hartman(14) emphasized the important role of bacteria in the production of methemoglobinemia. They studied the gastrointestinal flora with regard to nitrate reduction, gastric acidity, age and the level of intestinal absorption of nitrate. It was found that all micro-organisms isolated from the mouth and from the gastrointestinal tract were capable of reducing nitrate to nitrite and grew in media of pH 5-7.

The invading bacteria must adapt themselves to nitrite formation if they were not previously in contact with nitrate. Consequently, they felt it may take from four to five days after the first ingestion of nitrate until the full nitrate-reducing efficiency is reached. This may explain the commonly observed lag period of 1-3 weeks before the onset of the illness(12). However, in our own studies we found raised MetHb in infants 24 hours after exposure to powdered milk formula made with high nitrate water.

Gastric acidity: Examinations of gastric juice of infants who developed appreciable levels of MetHb revealed that the pH was usually higher than 4. It was found(15) that the normal pH of infant gastric juice varied between 3-5, but with unspecific diarrhea, gastric pH increased and ranged between 4.6 and 6.8; Mache(16) examined the pH and bacterial flora of gastric juice from children. They found the gastric juice was sterile at pH 4.6.

Gastrointestinal disturbances: It has been shown that all the members of the family Enterobacteriaceae are able to reduce nitrate to nitrite. Such organisms can gain access to the upper intestine during gastrointestinal disturbances(14). However, their ability to become established in the stomach is dependent on the pH, as mentioned in the preceding section. In the absence of nitrite-reducing bacteria in the stomach or upper intestine, most of the nitrate is probably absorbed as nitrate before reaching the colon in which the nitrate-reducing bacteria are normally found.

Type of powdered milk product: Studies in Czechoslovakia(8,11) indicated that the use of certain types of milk preparations has been suspected as the main cause for the development of methemoglobinemia in areas with high levels of nitrates in water. They reported on cases of methemoglobinemia in infants due to feeding with various brands of regular powdered milk which contained spores of B. subtilis, a nitrate-reducing bacteria; acidified milk powders which are often prepared by

fermentation with St. lactis did not cause any disease. The acidified milk preparation can contain an antibiotic substance called nisin which inhibits the growth of nitrate-reducing bacteria. On the other hand, Mucha et al(16) claimed that the main source of nitrate-reducing microorganisms could be eliminated by providing bacteria-free food to infants; however, under certain conditions, the ascent of bacteria from the colon to the duodenum and stomach cannot be prevented. B. subtilis spores are, however, not destroyed by normal milk pasteurization and drying processes. Little nitrate was found in the milk of cows drinking water with up to 800 mg NO_3/l(17). Many hold the opinion that mother's milk or cow's milk cannot be a cause of methemoglobinemia.

High fluid intake: Infants with an average fluid intake would ingest several times more nitrate per gram of hemoglobin than an adult due to their higher fluid intake per unit of body weight(7). Burden(18), assuming that all nitrate is reduced to nitrite, sets the permissible level of nitrate for adults at 1,086 mg/l in England and 198 mg/l in the tropics; for infants the permissible levels would be 88 mg/l and 26 mg/l respectively. His estimates are based on 13.7 mgNO_3/kg as the maximum daily amount which can be tolerated without giving rise to toxic symptoms. The governing factor would be, in his estimate, the relative daily fluid intake which for infants would be 0.5 liter in England and 2.0 liters in the tropics.

Effect of nutrition: Food composition can also affect the severity of the illness. On the one hand, there are certain nutrients such as vitamin C that can cure or prevent methemoglobinemia. High vitamin C intake among infants in some areas may explain the scarcity of the disease even when waters rich in nitrates are consumed. On the other hand, certain vegetables such as spinach, rhubarb, etc., contain considerable amounts of nitrates. Several cases of nitrate poisoning in infants after eating spinach were reported(19). In our own studies in Jerusalem we detected as much as 4850 ppm of NO_3 and 233 ppm of NO_2 in a local variety of spinach(20). Such concentrations in food can become particularly toxic if enogenic bacterial activity converts most of the NO_3 to NO_2.

Fetal hemoglobin: Hemoglobin F is oxidized more readily to methemoglobin(21). The fact that blood of newborn babies consists of more than 80% hemoglobin F might explain their increased tendency to develop methemoglobinemia.

Methemoglobin reduction: Methemoglobin reduction velocity in the presence of lactate or glucose is lower in cord blood erythrocytes than in adult blood. The lower methemoglobin reduction velocity in cord blood is explained by a temporary deficiency of DPNH - the methemoglobin reductase cofactor(22) or by low activity of the enzyme itself. A positive correlation between DPNH diaphorase activity and methemoglobin reduction was shown in adult blood but not detected in cord blood(23).

OBJECTIVES

The following general questions served as guidelines in carrying out the project objectives of evaluating the suitability of the current nitrate standard in drinking water.

1. Can a dose-response relationship be established between intake of nitrates in drinking water and the development of raised methemoglobin levels in infants?

2. At what threshold level of nitrates in water is the first effect detectable?

3. What environmental, nutritional, physiological or genetic factors influence this relationship?

4. What is the health significance, if any, of chronic sub-clinical or slightly raised levels of MetHb?

5. Can new sensitive parameters be used to detect health effects due to exposure to nitrates other than raised MetHb levels?

6. Are there direct toxic effects of exposure to nitrates and/or nitrites other than raised MetHb?

PROGRAM

In order to answer the above questions an extensive series of field and laboratory studies was initiated. The field work included studies of some 3000 infants in various parts of the country exposed to various levels of nitrates in water and on various dietary regimes.

Laboratory studies on various aspects of acute and chronic toxicity were carried out in parallel. A number of special investigations on sensitive methods of evaluating toxic effects were initiated including behavioral and neurohistological effects.

REFERENCES

1. Bodansky, O., Methemoglobinemia and Methemoglobin-producing Compounds, Pharmacol. Rev., 3, 144, 1951.

2. Lee, D.D.K., Nitrates, Nitrites, and Methemoglobinemia. Environ. Res. 3:484-511, 1970.

3. Jaffe, E.R., The Red Blood Cell (C.W. Bishop, ed.), p. 397, Academic Press, New York, 1964.

4. Lemberg, R. and Legge, J.W., Hematin Compounds and Bile Pigments, p. 328. Interscience Publishers, Inc., New York, 1949.

5. Tepperman, J., Bodansky, O. and Jandorf, B.J., Effect of Paraminopropiophenone-induced Methemoglobinemia on Oxygenation of Working Muscles in Human Subjects, Am. J. Physiol., 146, 702, 1946.

6. Ferrant, M., Methemoglobinemia: Two Cases in Newborn Infants Caused by Nitrates in Well Water, J. Pediat., 29, 588, 1946.

7. Gross, R., Arch. Hyg., 148, 288, 1964.

8. Knotek, Z., and Schmidt, P., Pathogenesis, Incidence and Possibilities of Preventing Alimentary Nitrate Methemoglobinemia in Infants, Pediatrics, 34, 78, 1964.

9. Winter, R.F., Tardiff, R.G., and McCabe, L.J., Nitrate in Drinking Water. Jour. Amer. Water Works Assoc. 63:95-98, 1970.

10. Rosenfield, A.B. and Huston, R., Infant Methemoglobinemia in Minnesota due to Nitrates in Well-water, Minn. Med. 33, 787, 1950.

11. Schmidt, P. and Knotek, Z., Problems of Nitrate Food Methemoglobinemia of Infants in Czechoslovakia. Gig. i San., 31, 290, 1966.

12. Teerhaag, L. and Eyer, A., Nitrate in Trinkwasser, Off. Gesundheitsdienst, 20, 1, 1958.

13. Subbotin, F.N., The Nitrates of Drinking Water and Their Effect on the Formation of Methemoglobin, Gig. i San., 2, 13, 1961.

14. Cornblath, M. and Hartman, A.F., Methemoglobinemia in Young Infants, J. Pediatrics, 33, 421, 1948.

15. Marriott, W.M., Hartman, A.F. and Senn, M.J., Observations on the Nature and Treatment of Diarrhea and the Associated Systemic Disturbances. J. Pediatrics, 3, 181, 1933.

16. Mucha, V., Kamensky, P. and Keleti, J., Genesis and Prevention of Alimentary Nitrate Methemoglobinemia in Babies, Hygiene and Sanitation, 30, 185, 1965.

17. Davison, E., Nitrate Toxicity in Dairy Heifers, Dairy Science, 47, 1065.

18. Burden, E.H., The Analyst, 86, 429, 1961.

19. Sinios, A. and Wedsak, W., Die Spinatvergiftung des Sauglins, Dtsch. med. Wschr., 90, 1558, 1965.

20. Eisenberg, A., Wisenberg, E., and Shuval, H.I., The Public Health Significance of Nitrate and Nitrite in Food Products. Ministry of Health, Jerusalem, 1970.

21. Bui-Dozu, P.S. and Pick, C., Spontaneous Oxidation of the Chain of the Foetal Haemoglobin Component of Cord Blood, Biochim Biophys. Acta 115, 495, 1966.

22. Ross, J.D. and Desforges, J.F., Reduction of Methemoglobin by Erthrocytes from Cord Blood, Pediatrics, 23, 718, 1959.

23. Kamasawa, Y., Hattori, M., Kosaka, K. and Nakao, K., The Relationship of NADH-dependent Diaphorase Activity and Methemoglobin Reduction in Human Erythrocytes, Clin. Chim. Acta, 19, 524, 1968.

SECTION IV

MICROMETHOD FOR THE DETERMINATION OF METHAEMOGLOBIN IN BLOOD

INTRODUCTION

In connection with the study to determine methaemoglobin (MetHb) levels in infants exposed to high nitrate content in water the need arose for a precise and accurate micromethod which would assure adequate stability of the sample to allow a field survey to be conducted in communities distant from the laboratory.

The method of Evelyn and Malloy(1), which up to date has been the standard procedure for the assay of MetHb in blood, is considered not sufficiently sensitive to determine low concentrations of the pigment, present in normal blood samples and to differentiate between normal and slightly elevated levels which might be indicative of subclinical cases of methaemoglobinemia caused by nitrate ingestion. Due to the instability of the MetHb in the drawn blood sample it is usually recommended in normal clinical procedures to carry out the test as rapidly as possible. This is generally not feasible under field survey conditions.

The procedure presented below is a modification of the Evelyn and Malloy method aimed to augment its precision. The size of the blood sample is kept at a minimum so that finger-tip blood samples from infants should suffice. The procedure also assures sample stability.

PRINCIPLE

MetHb is reacted with cyanide and the change in light absorption at 632 nm is measured.

REAGENTS

1. $K_3Fe(CN)_6$-5% (w/v) in water. Store in a dark bottle. Prepare monthly.
2. Phosphate buffer - 0.5 M, pH 6.0.
3. Ferricyanide-phosphate mixture: 0.2 ml of $K_3Fe(CN)_6$ solution (reagent 1) and 2.5 ml of phosphate buffer (reagent 2) are made up to 50 ml with water. Prepare daily.
4. Sodium cyanide-10% (w/v). Prepare monthly.
5. Acetic acid-12% (w/v).
6. Neutralized cyanide solution. Mix 1.0 ml of reagent 4 with 0.9 ml of reagent 5. Use within 6 h.

INSTRUMENTS

1. Highspeed microcentrifuge, capacity 10,000 rev./min. The Sorvall SS-1 centrifuge or the Eppendorf Microfuge with the corresponding 1.5-2 ml-microtubes were used.

2. Microcuvettes with a path length of 1 cm, inside diameter 3-4 mm and a minimal working capacity of 0.6 ml. The Zeiss Mfg cuvettes were used.

3. A Zeiss PMQ II or another equivalent sensitive spectrophotometer adaptable for the use of microcuvettes.

METHOD

Pipette 200 µl of freshly drawn, heparinized blood into a 1.5-2.0 ml micro centrifuge tube and hemolyse by adding 550 µl of water. Mix. After 3 min, add 250 µl of phosphate buffer (reagent 2) and mix again. Unless test is completed within 30 min, cool hemolysed and buffered sample rapidly to 2° and keep refrigerated until assayed. Centrifuge at 10,000 rev./min for 15 min. Centrifugation at high speed is absolutely necessary. Transfer the completely clear supernatant into a small tube to the first (1) of two microcuvettes. Transfer to the second (2) cuvette 50 µl of the supernatant and mix with 550 µl of the ferricyanide-phosphate mixture (reagent 5). In this step the total hemoglobin in cuvette No. 2 is oxidized to metHb. Measure the absorbances A_1 and A_2 at 632 nm against air. Add 30 µl of the neutralized cyanide solution (reagent 6) to each of the two cuvettes. Mix gently and let stand for 1 min. Read again absorbances A_3 and A_4.

Calculation

$$\frac{A_1 - A_3}{(A_2 - A_4) \times 17} \times 100 = \text{MetHb, in percent of total hemoglobin.}$$

RESULTS

Precision

In Table 1 data on the precision of the method are presented. Replicate blood samples drawn from three normal adults were assayed. The concentration of MetHb was around 0.5% of the total pigment. The standard error was in the range 0.019-0.025.

Accuracy

The accuracy of the method was investigated by recovery experiments. A solution of MetHb was prepared from nitrite-treated erythrocytes with

a MetHb content of 96%(2). The assay was modified by incorporating into a series of microcentrifuge tubes, each one containing 100 ul of normal blood, increasing amounts of the methemoglobinemic solution which substituted part of the 550 ul of water used for hemolysis. The results are summarized in Table 2. It can be seen that 94-99% of the added "Nitrite-methemoglobin" is recovered.

Table IV-1 PRECISION OF METHOD

Subject	Number of Replicates	Mean Percent Methemoglobin	S.E.
A	10	0.49	0.030
B	7	0.38	0.019
C	6	0.63	0.025

Table IV-2 RECOVERY OF METHEMOGLOBIN ADDED TO HEMOLYSATES

Methemoglobin added*	Percent of total pigment Found	Expected	Recovery Percent
none	0.45	-	-
1.2	1.50	1.65	96.4
2.4	2.84	2.85	99.4
4.8	5.33	5.25	99.4
9.6	9.85	10.46	94.5

*The added methemoglobin was prepared from nitrite-treated erythrocytes.

Stability of MetHb

The stability of methemoglobin in blood samples was investigated in both whole and hemolysed blood from normal adults (Table IV-3). Storage of whole blood kept at room temperature (24°) or refrigerated at 2° or below, results in the reduction of MetHb to Hb. Hemolysates, held at 24° for few hours show a considerable increase to MetHb. However, in buffered hemolysates kept at 2° for not more than 24 h the rate of auto-oxidation of hemoglobin is insignificant.

Table IV-3 STABILITY OF METHEMOGLOBIN WITH TIME UNDER DIFFERENT CONDITIONS

Condition of preservation	Percent of Methemoglobin at zero time	after 24 hrs
Hemolysate (2°C)	1.2	1.3
Hemolysate (2°C)	4.8	4.7
Hemolysate (2°C)	43.1	41.0
Hemolysate (24°C)	1.2	24.5
Whole blood (2°C)	1.2	0.9
Whole blood (24°C)	1.2	0.8
Whole blood (2°C)	43.1	18.8

Conversion of hemoglobin to methemoglobin at low temperatures

Initial attempts to keep blood samples until assay in the frozen state (at -20°C) failed as they showed considerable increases in MetHb. This phenomenon has not been previously reported.

Table IV-4 OXIDATION OF HEMOGLOBIN DURING STORAGE AT -20°C

Conditions	Percent of methemoglobin 0	5 hrs	24 hrs
Whole blood	0.6	5.7	12.4
Hemolysate	0.5	-	4.1

As can be seen from Table IV-4 this is a progressive reaction with time. Controls run simultaneously at +3°C and -4°C (not frozen) did not show any change in MetHb level. This reaction did not occur after freezing at -192°C. The possibility that this oxidation occurred only during slow freezing at -20°C was considered. The results presented in Table IV-5 show that rapid freezing of blood samples in liquid air (-192°C) does not prevent the oxidations of samples held at -20°C. Samples frozen at -192°C and held at this temperature for 24 hours were stable showing no increase in MetHb. Storage of blood samples in liquid air was not considered practical under field conditions.

Table IV-5 THE EFFECT OF STORAGE AT LOW TEMPERATURES ON THE OXIDATION OF HEMOGLOBIN

Conditions	Percent of Methemoglobin 0	5 hrs	24 hrs
Freezing -20°C; held at -20°C	0.4	-	9.8
Freezing -192°C; held at -20°C	0.4	5.1	10.5
Freezing -192°C; held at -192°C	0.4	-	0.4
Freezing -192°C; held for 3 hrs. at -20°C and there for 21 hrs at -192°C	0.4	-	4.8

Freezing experiments done in the presence of dimethylsulfoxide and concentrated sucrose solutions (results not presented) prevent (P) oxidation at -20°C. Nitrogen atmosphere during freezing at -20°C did not influence the reaction. The mechanism involved in this phenomenon is still obscure. Intracellular substrate may be involved in this reaction and conformational changes may be part of the mechanism. More experiments should be done before any conclusion can be drawn.

DISCUSSION

The sensitivity of the Evelyn and Malloy method in which the blood is diluted 1:100 was assessed by the authors to be sufficient to detect a minimum of 0.2 g MetHb per 100 ml of blood(3). This sensitivity is

considered low for the assay of MetHb in normal blood samples containing approximately 0.15 g MetHb per 100 ml (calculated on the basis of total Hb of 15 g per 100 ml wherein the MetHb content is 1%). By using a considerably more concentrated hemolysate with a dilution ratio of 1:5 in the modification prevented above, sensitivity was increased to detect approximately 0.02 g of MetHb per 100 ml.

The stability of the MetHb of the hemolysed freshly drawn blood cooled to 2° allows the carrying out of field surveys in communities distant from the laboratory, providing the samples are assayed within 24h; the pH of the buffered hemolysate should be approximately 7.35 (at 20°). Its alkalinity retards the auto-oxidation of Hb to MetHb. If the samples are stored at room temperature, they should nevertheless be assayed within 30 min.

REFERENCES

1. Evelyn, K.A., and Malloy, H.T. Micromethod of Oxyhemoglobin, Methemoglobin and Sulfhemoglobin in a single sample of blood, J. Biol. Chem. 126:655, 1938.

2. Rossi-Fanneli, A., Antonini, E., and Mondivi, B. Ferrihemoglobin reduction in normal and methemoglobinemic subjects. Clin. Chim. Acta. 2:476, 1957.

3. Henry, R.J., Clinical Chemistry, Harper and Row, Philadelphia, 1964, p. 757.

SECTION V

THE DETERMINATION OF NITRITE IN BLOOD

Investigations carried out in our laboratory concerning the relationship between the amount of nitrate in drinking water and the content of methemoglobin in blood emphasised the need for a method of estimating low nitrite levels in the circulatory blood of laboratory animals fed with nitrate or nitrite. The method was also required for planned field studies of infants consuming water high in nitrate concentration.

The existing methods for nitrite determination in blood(1-3) were not found to be satisfactory for the determination of very low concentrations of nitrite, especially in the small blood sample volumes available in these investigations.

In this work, different factors were investigated that may influence nitrite determination in blood by a spectrophotometric method, based on diazotisation and coupling reactions, involving sulphanilic acid and Cleve's acid. The modifications and refinements developed in order to reach a detection limit of 0.01 µg N in 0.1 ml blood are described in this paper. The advantages of the micromethod presented are high selectivity, wide range, accuracy and lack of photosensitivity of the final color obtained.

EXPERIMENTS

Apparatus

A Zeiss PMQ II spectrophotometer equipped with 10-mm cells (Helma 105/1 microcell) and an Eppendorf Microfuge 5200 centrifuge providing 15,000 rev min^{-1} with the special 1.5-ml microtubes were used. A conventional clinical centrifuge may also be used.

Reagents

All chemicals used were of analytical-reagent grade. Nitrite-free distilled water was used in the preparation of all solutions.

Sulphanilic acid solution. Dissolve 0.5 g of sulphanilic acid in 150 ml of a 30% (v/v) solution of glacial acetic acid in water. Store in a brown bottle.

Cleve's acid solution. Dissolve 0.2 g of Cleve's acid (1-naphthylamine-7-sulphonic acid) in 120 ml of water, warming in a water-bath. Filter the solution, cool and add 30 ml of glacial acetic acid. Store in a brown bottle in a refrigerator.

Stock nitrite solution. Dissolve 0.4928 g of sodium nitrite and dilute to 1 liter with water (1 ml = 100 µg of nitrite as nitrogen). Preserve with 1 ml of purified chloroform. Interfering substances present in the chloroform can be removed by extracting 100 ml of chloroform with four 20-ml portions of 0.1 M hydrochloric acid.

Standard nitrite solution. Dilute 10 ml of stock solution to 1 liter (1 ml = 1 µg NO_2-N). Prepare this solution immediately before use.

Zinc sulphate solution. Dissolve 4.31 g of zinc sulphate heptahydrate in water and make up to 100 ml.

Recommended procedure

Collection of samples. Adequate precautions must be taken during collection of blood samples for the determination of nitrite because it is rapidly oxidisable in vitro. Blood samples should be analysed within the shortest possible time. However, the sample may be stored at 4° for about 1 h without significant loss of nitrite content.

Deproteinization. Add 0.1 ml of blood to a 1.5 ml microtube containing 0.6 ml of zinc sulphate solution. Add 0.4 ml of bidistilled water and mix well, preferably using a Vortex-Genie apparatus. To this add 0.1 ml of aqueous 4% (w/v) sodium hydroxide solution and mix again. Keep on ice for 1 h. Remove the precipitated proteins by centrifugation (2 min at 15,000 rev min^{-1}).

Color reaction. Take 0.6 ml of clear supernate in a 100/10 test-tube and add 0.8 ml of bidistilled water; mix well, add 0.1 ml of sulphanilic acid solution and leave on ice for 15 min for the diazotization to take place. Add 0.1 ml of Cleve's acid solution and leave for 60 min at room temperature for the coupling reaction. If nitrite is present a red-violet diazo complex is formed. The absorbance is read at 520 nm.

Preparation of standard curve. Add several aliquots (up to 0.5 ml) of standard nitrite solution (1 ml = 1 µg NO_2-N) to Eppendorf centrifuge microtubes containing 0.6 ml of the zinc sulphate solution. Thereafter follow the technique as described under Recommended procedure.

Double-distilled water was substituted for nitrite solution for the reagent blank.

RESULTS AND DISCUSSION

The variables which may influence the results of nitrite determination in such a complex matrix as blood were investigated and different procedures for removing blood proteins were tested. The precision and accuracy of the method were evaluated statistically.

Removal of blood proteins

One of the problems of the method for nitrite determination in blood is the choice of a deproteinizing agent that will not inhibit color development. Most protein precipitants are active in acidic media, which are not suitable here, because of the instability of the nitrite ion. Therefore, only protein precipitants acting in slightly alkaline or neutral media may be considered. In the present work, two deproteinizing systems were compared: zinc sulphate with either barium hydroxide or sodium hydroxide. Figure V-1 shows the recovery curves between 0 and 0.5 µg of nitrite-nitrogen in standard solutions and after treatment with both the above-mentioned protein precipitants. It appears that the zinc sulphate-sodium hydroxide deproteinizing system is superior to the other system and does not inhibit color development.

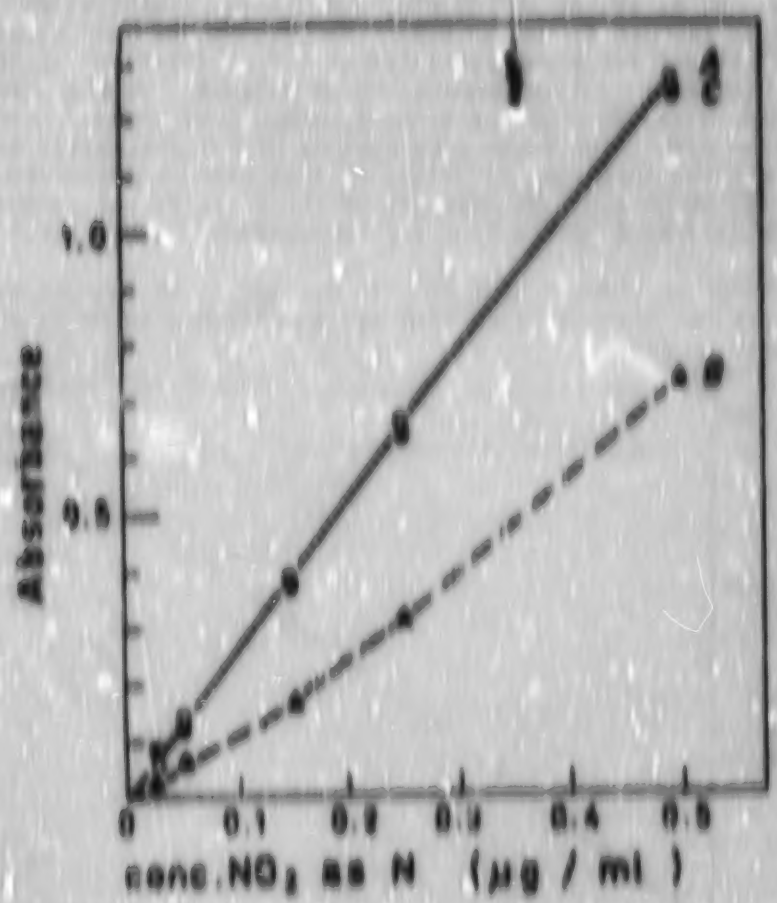


FIGURE V-1. INFLUENCE OF DEPROTEINIZING REAGENTS ON NITRITE RECOVERY (A) None; (B) $Ba(OH)_2 ZnSO_4$; (C) $NaOH ZnSO_4$

The color reaction

Coupling reagent. The classical coupling reagent in the Griess-Ilosvay reaction is α-naphthylamine(4). This substance has been shown to be carcinogenic(5). Among the other known non-carcinogenic coupling agents, the following two were chosen for investigation: 1-naphthylethylenediamine(6) and 1-naphthylamine sulphonic acid (Cleve's acid(7)). A comparison of these two coupling agents under the proposed experimental conditions showed that both reagents gave good results, but Cleve's acid was preferable; it provides a color of higher intensity, Beer's law is obeyed over a wider range and the color is less dependent on the temperature. The results are presented in Figure V-2.

Diazotization time and coupling time: The time factor is important in the reactions involved in this method. Different diazotization times were tried: 0, 5, 10, 15 and 20 min; and the coupling time was varied concomitantly between 30 and 90 min. The results obtained under these various conditions for a concentration of 0.2 μg NO_2-N per ml are presented in Figure V-3 and are summarized as follows: with a diazotization time of zero, when the coupling agent is added immediately after the diazotization agent, the color developed is of low intensity, and increases with time without levelling off after 60 min; with diazotization times of 5, 10, 15, or 20 min, the strength of the color formed increases with the increasing diazotization time and becomes more stable after 40-60 min.

Diazotization times longer than 15 min led to no further improvement. Thus, the optimal diazotization time was established to be 15 min and the coupling time 60 min.

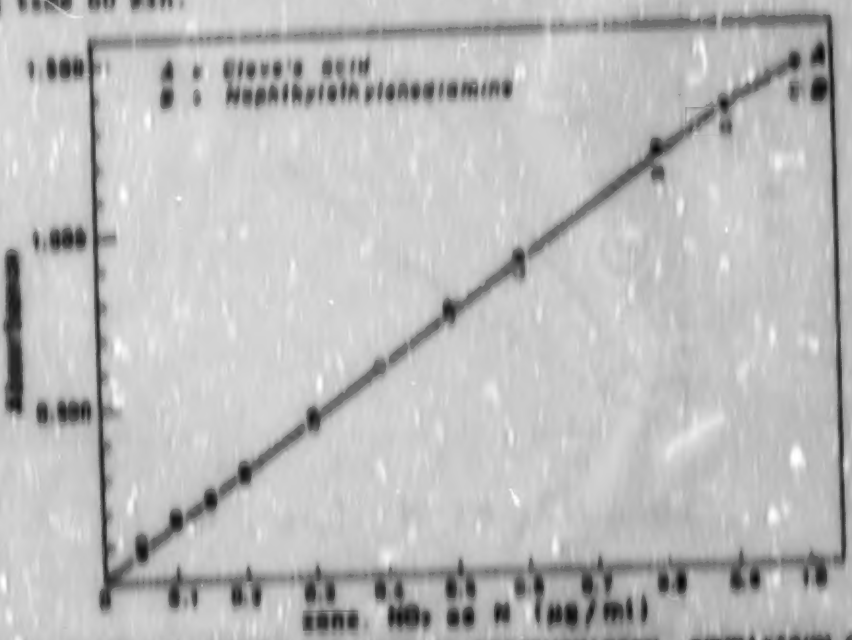


FIGURE V-2. STANDARD CURVES FOR NITRITE DETERMINATION, COMPARISON OF TWO COUPLING AGENTS, (a) Cleve's Acid; (B) Naphthylethylenediamine

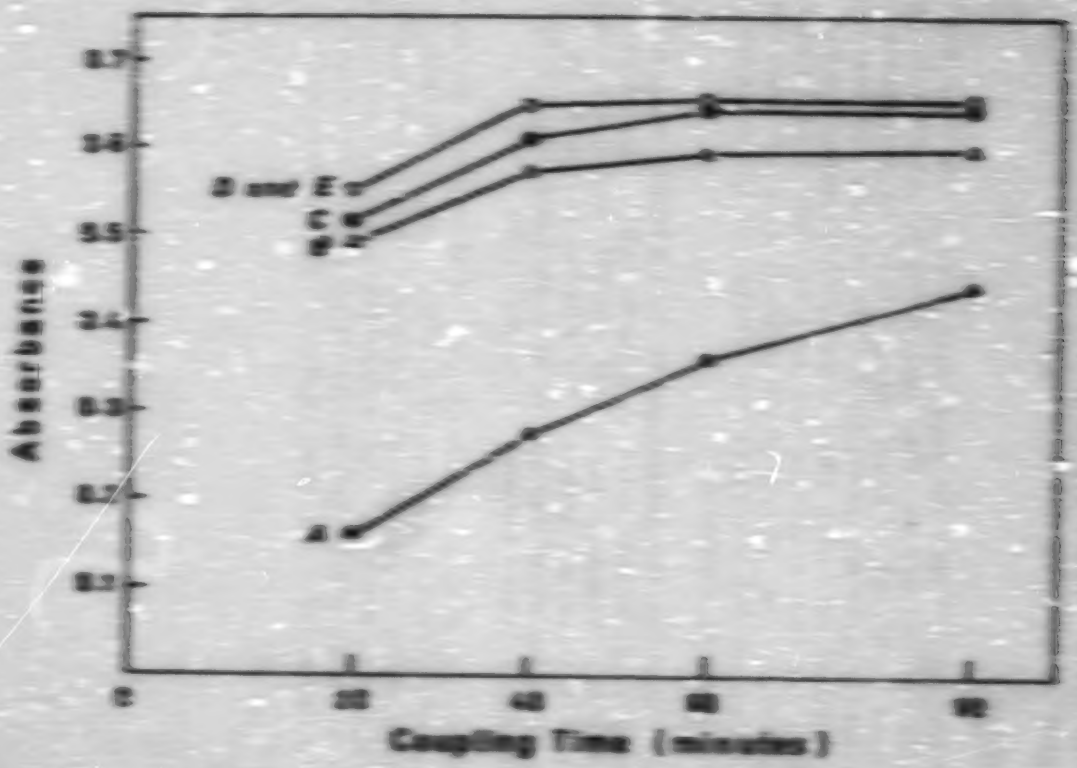


FIGURE V-2. EFFECT OF [illegible] TIME AND [illegible] TIME. [illegible] TIME: (A) 0 min; (B) 5 min; (C) 10 min; (D) 15 min; (E) 20 min.

The effect of temperature. The effect of temperature on the final color development was studied. During the diazotization time a temperature of about 1° is necessary. When the diazotization is done at 25°, the final absorbance is decreased by about 10%. During the coupling time, temperature variation between 20° and 30° does not affect the intensity of absorbance. In parallel determinations with naphthylethylenediamine and Cleve's acid, the influence of temperature was found to be somewhat greater with the former reagent.

Evaluation of method

Limit of detection. Under the experimental conditions and with the above-mentioned optical system, the limit of detection is 0.01 ug of nitrite-nitrogen in 0.1 ml of blood, which yields an absorbance of 0.012. This sensitivity was adequate for the research on residual nitrite in the blood of rats with induced methemoglobinemia. Other known methods(1-3) are less sensitive and need at least 1 ml of blood for each determination. The method of Litchfield(3), which requires less than 1 ml of blood, has a sensitivity similar to the proposed method, but needs special apparatus.

Precision. Precision was determined by 36 replicate analyses of standard solutions in distilled water with 1, 4, and 5.5 ug of nitrite-nitrogen per ml. Standard deviations and 95% confidence limits were calculated at the various levels. The results are shown in Table V-1. The standard deviation varied between 0.015 and 0.110, and the 95% confidence limits between ±0.01 and ±0.06.

Accuracy. The accuracy of the method was evaluated by determining the recovery of different amounts of nitrite added to plasma. Recovery data from whole blood were not evaluated because hemoglobin reacts immediately with nitrite. Ten determinations were carried out at each concentration in plasma and in distilled water; the results are presented in Table V-2. The mean recovery varied between 97.5 and 100.2%.

Table V-1 PRECISION OF THE METHOD FOR NITRITE DETERMINATION IN BLOOD

(12 Determinations were done at each level)

NO_2^--N Taken (ug ml^{-1})	Mean Value Found (ug ml^{-1})	Range	Standard Deviation	95% Confidence Limit
1.00	0.99	0.97-1.04	0.035	0.99 ± 0.01
4.00	3.99	3.96-4.04	0.035	3.99 ± 0.01
5.00	5.52	5.40-5.68	0.110	5.52 ± 0.06

Table V-2 RECOVERY OF NITRITE FROM BLOOD PLASMA

(10 Determinations were done at each level)

NO_2^--N Added (ug ml^{-1})	Mean NO_2^--N Found (ug ml^{-1})	Standard Deviation	Mean Recovery Percent	Recovery Range Percent
0.40	0.39	0.09	97.5	92-112
1.00	0.99	0.04	99.0	95-103
3.50	3.48	0.07	99.4	96,104
4.00	4.01	0.08	100.3	99,102
6.00	5.95	0.16	99.1	96,102

Some applications of the method

The proposed method was specially developed for the determination of small amounts of free nitrite in the blood of experimentally nitrite-intoxicated rats in connection with the levels of methemoglobin formed. Since hemoglobin reacts with nitrite, the determination of residual nitrite in blood is a necessary parameter in the study of this reaction. At the first contact between nitrite and blood, some nitrite reacts and hemoglobin is partially converted to methemoglobin. The partition of residual nitrite among the different blood components is a particular feature of interest.

Different amounts of nitrite-nitrogen between 1 and 100 μg ml⁻¹ were added to human blood in vitro and simultaneously, for reference, to distilled water. After the blood had been mixed, part of it was centrifuged and the plasma separated. Residual nitrite was determined in total blood and, in parallel, in plasma. The results obtained are presented in Table V-5. With nitrite concentrations varying between the above limits, the initial drop of nitrite in blood represents 76-90% of the amount added. The concentration of nitrite in plasma was about twice that found in blood. In Table V-5 the amount of nitrite was calculated for 0.55 ml of plasma, since plasma represents 55 ± 5% of the total blood volume(8). The total residual nitrite in 0.55 ml of plasma indicates figures very close to those obtained in 1 ml of whole blood. Therefore, it can be assumed that most of the residual nitrite in blood is bound in the plasma fraction.

Table V-5 RESIDUAL NITRITE PARTITION IN BLOOD AND PLASMA

NO_2^--N Added to Blood (μg ml⁻¹)	NO_2^--N Found: In Blood (μg ml⁻¹)	In Blood (percent)	In Plasma (μg ml⁻¹)	In Plasma (μg 0.55 ml⁻¹)
100.0	24.0	24.0	45.0	24.45
60.0	15.0	25.0	25.0	13.80
30.0	7.0	23.3	14.0	7.60
21.0	4.0	19.0	7.8	4.10
10.0	1.8	18.0	3.6	1.90
1.0	0.1	10.0	0.2	0.11

Plasma represents 55% of the total blood volume 15%.

REFERENCES

1. Weiglitz, E.J., and Palmer, A.C. "A Colorimetric Method for the Determination of Nitrite in Blood," J. Pharmacol. Exp. Ther. 11, 498, 1954.

2. Diven, R.H., Lister, W.J., Reed, R.E., Trautman, E.J., and Watts, R.E. "The Determination of Serum or Plasma Nitrate and Nitrite," Am. J. Vet. Res. 23, 24, 1962.

3. Litchfield, M.A. "The Automated Analysis of Nitrite and Nitrate in Blood," Analyst 92, 152, 1967.

4. Standard Methods for the Examination of Water and Wastewater, 12th Ed., American Public Health Association, New York, p. 205, 1965.

5. Precautions for Laboratory Workers who Handle Aromatic Amines, Chester Beatty Research Institute, London, 1958.

6. Saltzman, B.E., "Colorimetric Microdetermination of Nitrogen Dioxide in the Atmosphere," Anal. Chem., 26, 1949, 1954.

7. Bunton, N.G., Grosby, N.T., and Patterson, S.J. "Factors Influencing the Colorimetric Determination of Nitrite with Cleve's Acid," Analyst, 94, 585, 1969.

8. Wintrobe, M.M. "Clinical Hematology," 6th Ed., Lea Febiger, Philadelphia, p. 86, 1967.

SECTION VI

DETERMINATION OF NITRATE IN HIGH MINERALIZED DRINKING WATER USING THE ION SELECTIVE ELECTRODE

INTRODUCTION

A great variety of methods for nitrate determination in water have been described. The majority of them fall into the following types: nitration by the nitrate radical(1), reduction of the nitrate radical(2), oxidation by the nitrate radical(3), ultraviolet absorption(4) and polarographic technique(5). Several serious interferences are commonly encountered and they are often time consuming. All these methods are presented in "Standard Methods for the Examination of Water and Wastewater"(6) and it is not surprising that all the methods are still listed as tentative ones, because the nitrate determination is one of the most difficult tests and its accurate measurement remains yet one of the unsolved problems in environmental chemistry.

During the past few years an electrode selective for nitrate ion has been developed, which is intended to give a direct measure of the concentration of nitrate ion in solution. Pattersen and Shults(7) have reported on the theory and operation of this electrode. The selective ion electrode technique for nitrate analysis has already found widespread use in both laboratory and field determinations(8-10). It has been used in soil and plant extracts(11,12), in microbial media(13), in measuring sodium nitrite as an impurity(14) and in some aqueous systems (15,16). The nitrate selective electrode appears to be convenient as a simple and rapid method for determining nitrates in water.

The object of this study was to establish the analytical efficacy of the nitrate specific electrode in analysis of some drinking waters, characterised by high mineralization and large variations in concentration of interfering ions. The electrode measurements were compared with results of the phenoldisulphonic acid method, the most commonly used spectrophotometric standard procedure.

EXPERIMENTAL

Instruments

Potential measurements were read from a battery powered pH/mV specific ion meter (Orion Model 401). Both absolute and relative millivolt modes are provided. The nitrate indicator electrode was an Orion

Model 9207 and the reference electrode was a single function electrode Orion Model 9001 filled with the reference solution described later.

A Zeiss PMQ II spectrophotometer with 1 cm cells was used for measurements in the phenol disulfonic acid method.

Reference Electrode

The reference electrode filling solution was prepared from a saturated sodium sulphate solution, stirred and filtered. The original Orion filling solution 90000U1, provided with the reference electrode, contains nitrate ion(17) and it was proved unsuitable because contamination of the test solutions may occur particularly when measuring dilute samples. The composition of the electrolyte of the reference electrode causes interference if it contains ions for which the ion selective electrode is sensitive. Recently Orion recommended a double function reference electrode bridged with saturated sodium sulphate solution(18). The use of specific ion fluoride electrode as a nitrate ion reference electrode was also reported(19).

Reagents

All reagents were of analytical reagent grade. Distilled water was always glass distilled.

Sodium nitrate stock solution 200 ppm nitrate as nitrogen. Weigh 1.2140 gr. sodium nitrate which has been previously dried at 120°C. Transfer to a 1 liter volumetric flask, dissolve. Add 1 ml of 0.1% phenylmercuric acetate preservative solution, to inhibit biological growth.

Sodium nitrate standard solutions were prepared by diluting appropriate amounts of stock solution. All diluted standards must be prepared daily.

Sodium sulphate stock solution 4000 ppm as sulphate. Add 5.9154 gr sodium sulphate to a 1 liter volumetric flask. The conductivity of this solution is 3.4 mmhos. Dilute the stock solution as needed.

Interfering anions solutions. - Chloride, carbonate, bicarbonate, nitrite, sulphate, all sodium salts, were used as sources of interfering anions.

METHOD

Calibration

For greater precision in reading electrode potential the expanded M.V. position ("M.V. EXP") is used. Measurements made on this scale are

relative and can be adjusted with the calibration control. The lowest concentrated nitrate standard is set to give a near full scale positive reading.

For drinking water the maximum recommended level is 10 ppm nitrate as nitrogen; the scale meter is calibrated with 10 ppm nitrate-nitrogen in the middle and the upper point is set on 100 ppm. The lower limit should then be 1 ppm, since the meter covers a range of two logs in concentration at any one setting. The potential of each standard solution containing 1.0, 5.0, 10.0, 25.0, 50.0 and 100.0 ppm nitrate-nitrogen was measured by transferring about 50 ml of solution, respectively, into a 100 ml beaker, immersing the electrodes and stirring uniformly with a small teflon stirring bar for one minute before reading the mV value. A high degree of uniformity in stirring and temperature for all standard solutions is critical. In this study all determinations were made at 23° C ± 1°. Temperature fluctuations due to heat from stirring motor were eliminated by using a thermal insulating barrier between the beaker and the stirrer.

The relationship between nitrate concentration of the standardizing solutions and the equilibrium cell potential E real is plotted on semilog paper, the values of the concentration in ppm being plotted on the log axis. Absolute calibration curves (real millivolt scale) are used to verify correct electrode operation.

Sample Analysis - Taking into account both ionic strength and interfering anions, the procedure for nitrate analysis with the electrode is as follows:

The concentration of chlorides is determined and the chlorides are removed from solution by quantitative precipitation with silver sulphate.

The ionic strength of the unknown sample is estimated from specific conductivity measurements.

Before reading the potential of sample solution or a group of samples with about the same ionic strength, calibrate the meter with two or three standard nitrate solutions differing in concentration by a factor of 10 (1 ppm, 10 ppm and 100 ppm nitrate-nitrogen) and having the same total ionic strength as the unknown sample. This can be done by diluting 1:1 standard nitrate solution with sodium sulphate solution, prepared in the needed concentration to obtain the same conductivity in standard as in sample. (In preparing all dilutions pipets and volumetric glassware must be used). Immerse electrodes successively in each standard calibration solution and adjust with Calibration Control so that potential reading will correspond exactly to the calibration curve millivolt previously obtained for the respective standard. Finally, place the electrodes in the unknown sample and read, at the same position, the potential developed.

Allow reading to stabilize after one minute stirring, at the same rate and temperature as the standard. The potential measurement of

the sample is converted to nitrate concentration by using the standard calibration curve. Between measurements electrodes should be blotted dry with absorbent tissue.

RESULTS AND DISCUSSIONS

Interfering Factors

The measured potential of the nitrate activity depends on the ionic strength of the solution because the activity coefficient decreases with increasing ionic strength. On the other hand, electrode response is increased by interference from many common anions and this increased potential can counteract the influence of the ionic strength. The net interference is the result of these two opposing effects.

Ionic Strength : In principle the influence of ionic strength can be avoided in electrode determinations by the addition of a relatively concentrated salt solution of a non-interfering electrolyte, such that all the samples and standards are analysed at the same ionic strength.

Sodium sulphate solution is the best ionic strength adjuster because it has the properties of high ionic strength, good equitransference and is free of nitrate interfering anions, its selectivity constant being $K = 5.10^{-5}$ (18). The ionic strength of samples can be estimated by measuring the electrical conductance. In standard and unknown solutions of the same ionic strength, nitrate ion activity is assumed to be in constant proportion to concentration. In practice, this assumption involves determination first of the conductivity of the unknown sample /solution/ then calibration of the electrode with a variety of mixed standard solutions prepared in sodium sulphate solution with a concentration of identical conductivity value as the sample.

The effect of total ionic strength has been verified by the increase in slope of the calibration line at high salt concentration and by increase in nitrate content found, when measurements are made in the same diluted solution.

Table VI-1 presents results obtained for a solution of 10 ppm nitrate-nitrogen and varying sodium sulphate concentrations. The calibration was made with 500 ppm sodium sulphate solution containing various amounts of nitrate. The measured nitrate content is smaller when the sulphate concentration increases; this is the effect of ionic strength, which influences the activity of nitrate ion. The interference from sulphate anion is insignificant.

Anion Interference : Theoretically the magnitude of anions interference can be calculated by substituting the concentrations (Ci) and

Table VI-1. DETERMINATION OF NITRATE BY SELECTIVE ELECTRODE METHOD AT VARIOUS IONIC STRENGTHS

Nitrate nitrogen added ppm	Sodium sulphate ppm	Conductivity mmhos cm^{-1}	Nitrate nitrogen detected by electrode ppm
10	0	0.065	11.4
10	50	0.135	11.2
10	200	0.300	10.5
10	500	0.600	10.1
10	1000	1.100	9.0
10	2000	1.900	8.3

Table VI-2. ANIONIC INTERFERENCE IN NITRATE-NITROGEN DETERMINATION BY SPECIFIC ION ELECTRODE

		Nitrate - as nitrogen added, ppm					
Anion added, ppm		0	1	5	10	25	50
		Nitrate as nitrogen detected, ppm					
	5	1.20	1.30	5.00	10.10	26.10	55.10
Nitrite	10	1.60	1.70	5.60	10.50	28.20	58.10
	50	4.40	4.50	8.40	13.50	28.70	60.40
	50	0.25	1.10	5.80	11.50	25.00	50.00
Bicarbonate	250	0.40	1.50	7.30	12.00	26.10	51.50
	500	0.50	1.60	7.50	13.50	27.00	52.10
	50	1.00	1.50	6.00	11.40	27.00	54.00
Carbonate	250	1.15	1.70	6.40	12.00	29.00	58.00
	500	1.30	1.70	6.70	12.50	29.00	59.50
	50	1.30	1.30	5.80	11.50	27.00	52.00
	250	2.60	2.10	7.00	15.20	28.60	56.00
Chloride	500	3.80	3.00	8.00	15.20	30.00	59.10
	1000	5.20	4.50	9.50	17.00	34.00	64.70
	50	0.20	1.00	4.90	10.40	25.00	50.00
	200	0.30	1.10	5.00	10.10	25.10	50.00
Sulphate	500	0.60	1.00	5.00	10.00	24.90	49.70
	1000	0.70	0.90	4.80	9.60	24.90	49.30

selectivity constant (K_i) values of interfering anions (i) into the electrode response equation. The precise evaluation and empirical correction of anion interferences is difficult to establish, because the electrode response is influenced not only by the concentration of the interfering anion, but also by total solution compositions and by the nitrate level. Therefore the approach to a systematic correction has important limitations.

To evaluate the net influence of most common anions occuring in water, electrode measurements were made in a variety of synthetic solutions containing from 1 to 10 ppm nitrate-nitrogen and varying amounts of nitrite, hydrogen carbonate, carbonate, chloride and sulphate. The results are presented in Table VI-3.

It is observed that the level of interfering ion, the total ionic strength and the level of nitrate do influence the measured potential. For most anions occuring in water, additive influences from nitrite and chloride are major. Nitrite is seldom present in high concentrations in drinking waters. As to the strongly interfering chloride, it must be initially removed by precipitation with silver sulphate if accurate determinations are to be made. The elimination of chloride was also performed by treatment with cationic ion exchange resin, prepared in the silver form; in this study better results were obtained by Ag_2SO_4 precipitation.

Removal of interferences is a problem: an excess of removing reagent may increase sample ionic strength and may deviate the pH from the range of proper electrod response. To avoid this excess, it is necessary to know the concentration of interfering chlorides prior to their removal by precipitation. The advantage is that after this precipitation, sodium sulphate is formed in the sample medium solution - the same ionic strength adjuster as added in standard solutions prepared for calibration.

Langmuir and Jacobson(16) have calculated the approximate amounts of different anions which, if present together with 10 ppm of NO_3 produce a +1% error in the nitrate determination by electrode method. The most interfering anion was found to be nitrite; for this anion the presence of 1.2 ppm is sufficient to cause an error of +1%. The chloride anion causes a similar error when present in concentration of 2 ppm. The sulphate anion causes little interference. There must be 5000 ppm to cause an error of +1% in the 10 ppm NO_3 determination.

Operating Procedure Conditions

Temperature Effects - The Nernst equation is temperature dependent and the slope of the calibration plot E versus nitrate concentration changes with temperature. This dependence is not the same for different membrane materials, therefore a universal temperature compensator is not feasible. Precise results require meticulous temperature control and samples and standards must be measured at the same temperature.

Electrode Equilibration - The response time is mainly determined by the measuring conditions. Continuous and reproducible stirring of the samples and standards is necessary. Non-uniform stirring causes instability of the potential at equilibrium. The degree of stirring of the solution affects the equilibration time of the electrode; stirring of the solution reduces the time taken to reach equilibrium. High stirring rates introduce air bubbles into the solution, which cause unstable electrode potential response, because the air bubbles accumulate on the surface of the electrode and decrease contact with the solution. In the usual standardised conditions equilibrium is reached after 1 minute.

The depth of immersion of the electrodes in solution must always be the same. On changing over from one sample to another, the equilibrium at the surface has to be re-established; the time required for this depends on the difference between the concentrations in the samples. With large concentration jumps, when going from a high to a low concentration, more time is required before the measurement is stable.

Between determinations, electrodes should be wiped dry with absorbent tissue before immersion in the next solution.

Comparison with Phenoldisulfonic Acid Method

Parallel determinations of nitrates were performed on 148 spring and well water samples, by both the standard phenoldisulfonic acid method (P.D.S.) and the described specific electrode method. The ranges of nitrates as found by the P.D.S. method, varied between 1.4 to 40.7 ppm, most of the samples (78%) having between 5 to 20 ppm. The mineralisation of the samples varied largely and was high in most of them. The conductivity varied between 0.45 to 3.3 mmhos and the chlorides between 40 to 750 ppm; most of the samples (65.6%) contained more than 300 ppm chlorides.

The results of this comparison are summarised in Table VI-3. A good agreement was generally observed between the results of both methods. The correlation coefficient (r) for the relationship of phenoldisulphonic acid and electrode values was found .98. The linear regression equation is $y = 0.99x + 0.96$.

The electrode measurements values are in most cases higher than those of the phenoldisulphonic acid method. Perhaps this is because this procedure does not correct for bicarbonate interfering anion. The mean differences do not exceed 10%.

The results of nitrate determination by electrode method were compared with those of phenoldisulfonic acid method, as a function of con-

Table VI-3. COMPARISON OF SPECIFIC ION ELECTRODE AND PHENOLDISULPHONIC ACID METHOD FOR NITRATE-NITROGEN DETERMINATION IN DRINKING WATER SAMPLES

Nitrate-nitrogen level range ppm	Number of samples	Nitrate-nitrogen, ppm Electrode mean values	Nitrate-nitrogen, ppm P.D.S.* mean values	Conductivity, mmhos. Min.	Conductivity, mmhos. Max.	Chlorides ppm Min.	Chlorides ppm Max.
< 5.0	4	2.92	3.63	0.45	1.10	76	140
5.0 -10.0	46	6.78	8.03	0.50	3.15	40	50
10.1 -20.0	71	15.34	14.45	0.70	3.15	411	740
20.1 -30.0	12	26.12	25.00	0.80	2.70	95	600
>30.0	10	35.34	34.86	1.80	7.00	500	580
TOTAL	143						

*Phenoldisulphonic Acid

Table VI-4. THE MEAN DIFFERENCES (PERCENTAGE) BETWEEN THE RESULTS OBTAINED BY P.D.S.* AND E* METHODS, CORRELATED WITH LEVEL OF NITRATE AND CONDUCTIVITY VALUES

Nitrate-nitrogen level range ppm	Number of samples	Mean difference percent	Number of samples	Mean difference percent
1.4 - 9.9	39	6.6	10	20.1
10.0 -19.9	25	5.0	46	7.4
20.0 -40.7	5	4.0	33	3.5

*P.D.S. phenoldisulphonic acid
E electrode

ductivity and of nitrate level. The percentage differences between the two methods are presented in Table VI-4. The differences are more accentuated in the samples with low nitrate content (<10 p.p.m) and high salt content, (conductivity > 2.0 mmhos.) The differences are lower in samples with high nitrate concentration.

CONCLUSIONS

The results obtained in nitrate determination in highly mineralized waters by the electrode method performed under meticulously controlled laboratory conditions including removal of chlorides are in good agreement with those obtained by the standard phenoldisulphonic acid method. The electrode measurements values are in general somewhat higher than those of the phenoldisulphonic acid method, especially in the range of low nitrate concentrations and high salt contents.

The electrode method for nitrate determination is not a field method, because of detailed calibration requirements and the complex controls required to obtain reliable results. When nitrate concentrations are low and chlorides are high the electrode results may be many fold higher than in reality.

In water of low mineralization it may be used as a screening method to identify the content of nitrate with a minimum of special procedures.

In water of high mineralization it needs standardisation of curves with solutions of the same order of ionic strengths as the samples and elimination of interfering anions. Therefore, it is laborious when different types of water are to be examined in the same series.(10)

The electrode method is practical and rapid, when many samples belonging to a same source are to be compared or for continuous monitoring of a water supply with known mineralization. It cannot be relied upon as a field method of universal applicability unless degree of accuracy required can allow for deviations of some 20%, or more.

REFERENCES

1. Tarns, M.J. Phenoldisulphonic Acid method of determining nitrate in water. Anal. Chem. 33:1020, 1960.

2. Edwards, G.P. Determination of nitrates in wastewater effluents and water. J.W.P.C.F. 34:1113, 1962.

3. Jenkins, D. & Medsker, L.L. A brucine method for the determination of nitrate in ocean, estuarine and fresh waters. Anal. Chem. 36:610, 1964.

4. Navone, R. Proposed method for nitrate in potable waters. J.A.W.W.A. 56:781, 1964.

5. Frazier, R. E. Polarographic determination of nitrate nitrogen in water. J.A.W.W.A. 55:624, 1963.

6. Standard Methods for the Examination of Water and Wastewater 13th edn. APHA, AWWA and WPCF New York, 1971.

7. Potterton, S.S. & Shuitz, W.P. Analyt. Lett. 1, 11, 1967.

8. Andelman, J.B. Ion Selective Electrodes theory and applications in water analysis. J.W.P.C.F. 40:1844, 1968.

9. McClelland, N. & Nancy, E.H. (1972) Water Quality monitoring in distribution systems. J.A.W.W.A. 12, 705.

10. Durst, R.A. editor, Ion Selective Electrodes. National Bureau of Standards Special Publication 314, 1969.

11. Olsen, A. & Selmer Olsen, A.R., Nitrate determination in soil extracts with nitrate electrode. Analyst 94:886, 1969.

12. Paul, J. L. & Carlson, R.M., Nitrate determination in plant extracts by the nitrate electrode. J. Agr. Food Chem. 16:637, 1968.

13. Hensman, S.E., Nitrate ion selective electrode in microbial media. Applied Microbiol. 18:479, 1969.

14. Gehring, G.D., Dippel, W.A. & Soucher, R.S., Determination of sodium nitrate in sodium nitrite by selective ion electrode measurement. Anal. Chem. 14:1686, 1970

15. Keeney, D.R., Byrne, R.H. & Gensen, J.J., Determination of nitrate in waters with the nitrate selective ion electrode. Analyst 95:385, 1970.

16. Langmair, D. & Jacobson, D.L., Specific ion electrode determination of nitrate in some fresh waters and sewage effluents. Env. Sci. Tech. 4, 834, 1970.

17. Orion Research Inc., (1970) Instruction sheet single function reference electrode.

18. Orion Research Inc., (1972) Analytical methods guide. Fourth edn.

19. Manahan, S.E., Anal. Chem. 42:128, 1970.

20. Shechter, H. & Gruener, W. An evaluation of the ion selective electrode method for determination of nitrate in highly mineralized drinking water. J.A.W.W.A. 68:543,1976.

SECTION VII

MEASUREMENT OF ASCORBIC ACID IN BLOOD

INTRODUCTION

Ascorbic acid is considered to be one of the natural antimethemoglobinemic agents and is also used clinically as therapeutic agent for that purpose(1).

The importance of this compound from an epidemiological and nutritional point of view has become apparent from our field studies(2).

In order to check ascorbic acid levels among infants which are the susceptible section in the population, we developed a sensitive micro-method for ascorbic acid in blood.

Existing methods for ascorbic acid measurement in blood suffer from several disadvantages:

1) Low sensitivity.
2) Lack of specificity.
3) Large volume samples.

PRINCIPLE

Ascorbic acid reduces Fe^{+3} to Fe^{+2} which is complexed by a specific iron reagent to a red compound which is determined by photometry.

REAGENTS

3M Acetate buffer pH 4.5.
10% Trichloroacetic acid (TCA).
0.05% Bathophenantroline disulphate (BFAS).
0.05% Ferric chloride.
25% Orthophosphoric acid.

METHOD

All reagents added consecutively, in order with thorough mixing at each addition.

1) 0.25 ml of TCA was added to 0.25 of clear plasma. 0.25 ml of TCA supernatant was used.

2) 0.25 ml $FeCl_3$.

3) 0.20 ml orthophosphoric acid.

4) 0.30 ml BFAS.

5) 0.20 ml acetate buffer.

Hold in dark. After 30 min. read against ascorbic acid standards (in 5% TCA) at range between 0-1 mg/100 cc. Blank contains 0.25 ml 5% TCA in place of supernatant. Read at 534 mμ in Coleman spectrophotometer. Linearity up to 10 mg% in plasma.

RESULTS

Table VII-1. HUMAN ASCORBIC ACID LEVELS

mg/100 cc of plasma

Source	Mean	N
Cord blood	1.73	130
Infants (1-5 months)	0.94	24
Pregnant women	1.08	33

The results represented here show that the fetus has a higher level of ascorbic acid than the mother and that this level fall to half within the first months of life. This fact correlates with our findings that Metth levels are low in the fetus and are the highest at 0-3 months then fall to adult level. This phenomenon may hint that ascorbic acid may be a factor in determining Metth levels *in vivo*.

COMMENTS

1) TCA did not have any effect in complex formation. Perchloric acid as precipitant can replace TCA.

2) Kinetic study on effect of time and temperature gave best linearity at 30-90 at 40°C.

When a water-bath is not available incubation can be at room temperature as well.

3) FeCl$_3$ optimal concentration was found to be 0.5%. Recovery of ascorbic acid based on Fe^{+3} concentration found under these conditions was 95%.

4) Highly cleaned laboratory glassware is absolutely necessary.

5) When whole blood was used a small steady increase was observed. This may be because of glutathion presence.

6) Storage of samples (Plasma or deproteinized plasma in TCA) did not show any loss for 24 hrs. for long storage freezing is recommended

REFERENCES

1. Tons, G. The Congenital Methemoglobinemia, Bibliotheca Haematologica No. 28, S. Kruger, Basel, N.Y., p. 72-74, 1968.

2. P'uval, H. T. and Gruener, N., Am. J. Pub. Health 63:1045, 1973.

SECTION VIII

EPIDEMIOLOGICAL STUDY OF THE ASSOCIATION BETWEEN NITRATES IN DRINKING WATER AND METHEMOGLOBIN LEVELS IN INFANTS REHOVOT AREA

INTRODUCTION

Infant methemoglobinemia resulting from consumption of water with high nitrate concentrations was first recognized clinically by Comly in 1945(1). Since then about 2,000 cases, including many fatal poisonings in infants resulting from ingestion of water containing nitrates have been recorded in various countries throughout the world(2). The U.S. Public Health Service Drinking Water Standards(3) recommend that water consumed by infants not contain over 45 mg/l of nitrates as NO_3. The World Health Organization has made a similar recommendation. These standards were originally based on limited epidemiological evidence gathered by Walton in 1951(4) indicating that no cases of methemoglobinemia had been reported in the United States when water containing less than 45 mg/l of NO_3 was consumed. More recent studies in Europe have, however, reported on clinical and subclinical cases of the disease in infants consuming water having less than 45 mg/l of NO_3(5).

Recently there has been pressure to relax the nitrate standard in drinking water in areas exposed to increasing nitrate pollution of ground water in which overt cases of infant methemoglobinemia are rarely reported. The Department of health in California has issued administrative orders which require surveillance of infants' health in communities supplying water containing up to 90 mg/l NO_3 while recommending the discontinuation of the source only at higher concentrations. The W.H.O. Recommended Drinking Water Standard for Europe-1970 more or less have followed suit. The 1971 International Drinking Water Standards of the W.H.O. retained, however, the recommended maximum limit of 45 mg/l. The standard set in Israel is 45 ppm NO_3 as the recommended limit but allow the use of water up to 90 ppm on condition that low nitrate water be made available to infants up to one year of age.

The epidemiological study reported upon here is part of a broad spectrum program to re-evaluate the current nitrate standard for water. Of particular interest was the possible presence of chronic sub-clinical methemoglobinemia in infants in the areas of Israel having medium-high concentrations of nitrates in the water supply but few overt clinical cases, as reported on by Znotek and Schmidt in Czechoslovakia(5).

METHODS

Two thousand four hundred seventy-three infants were studied in communities with medium-high and low concentrations of nitrates in the drinking water. The study area is on the coastal plain of Israel and

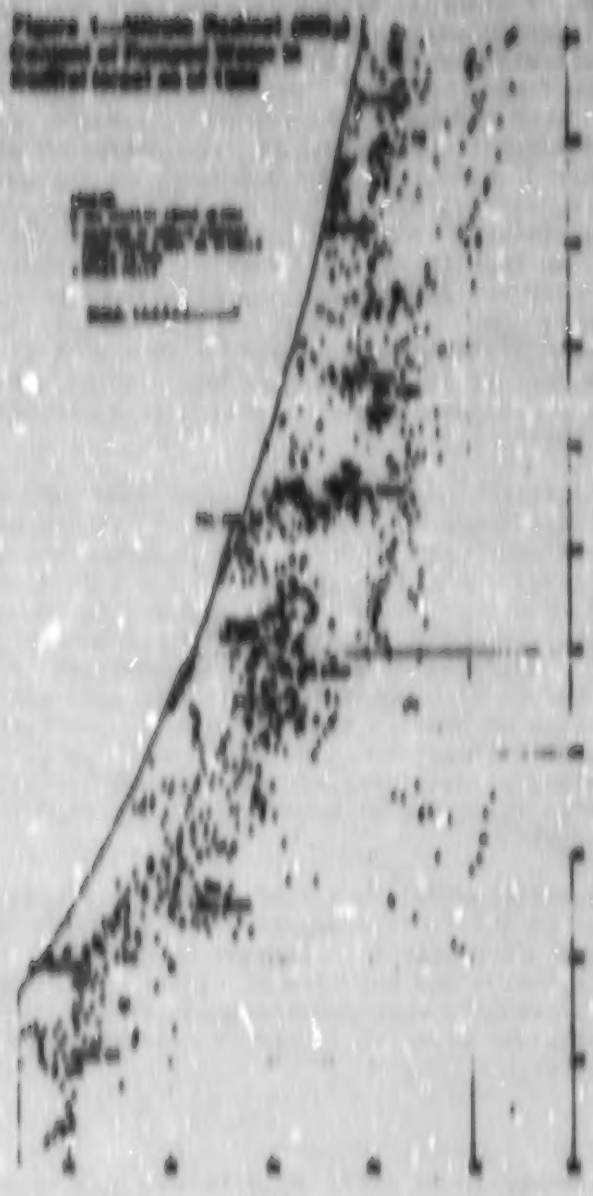


Figure VIII-1. Nitrate Radical (NO_3) Content of Pumped Water in Central Israel as of 1968.

includes the towns of Rehovot, Rishon le Zion, and Nes Ziona as well as a number of small agricultural settlements near Netanya (see Figure VIII-1). The nitrate concentration in most of the water supply wells in these communities varied from 50-90 mg/l. However, since the integrated water supply systems in each community are served by a number of wells of different nitrate content, the actual NO_3 concentration at the tap in a given home may vary from hour to hour depending on the pumping rates and water demand. Infants were examined at well-baby clinics run on a routine basis by the Ministry of Health, or by the Kupat Holim-Medical Insurance scheme, so that it was not practical to determine the exact concentration of nitrates at the home tap of the infant examined at the time of examination. For the purposes of this analysis all infants from the study area are considered as one group with a mean of about 70 mg/l. Jerusalem, with 5 mg/l of nitrates in the water supply, was chosen as a control area, and 756 infants were examined at Municipal Health Department well-baby clinics.

A 0.2 ml fingertip blood sample was taken from each infant studied by project staff nurses and examined for hemoglobin and methemoglobin (MetHb) in the laboratory in Jerusalem. An accurate and precise micromethod for testing MetHb was developed to meet the needs of this study and similar field studies. The method is a modification of the classical Evelyn and Malloy(6) method and has been reported upon previously(7). Blood samples are stabilized so that they can be taken in distant communities and transported to the laboratory for assay within 24 hours rather than within less than an hour as required by the conventional procedure. This method can show differences in MetHb as small as .1% which is essential in a study aimed at detecting slightly raised levels of MetHb in a healthy population where normal MetHb levels are expected to be about 1% of total hemoglobin.

A detailed questionnaire was filled in by the project nurse for each infant, based on information supplied by the mother and the files of the well-baby clinic (appendix I). Details on age, sex, weight, ethnic background, health status and nutritional regime were recorded and together with the laboratory results were punched on cards for computer analysis. Particular attention was given to intake of tap water with milk formula or from other sources.

RESULTS

There are essentially no differences between the mean MetHb levels in the study group and the control group as shown by Table VIII-1. The mean MetHb level for the entire population studied was 1.04% with a standard deviation of .72%. For the most susceptible age group of 1-60 days the mean MetHb for the study group is 1.36% while being 1.30% for the controls. The mean for both groups is 1.33%. The differences are not significant. For all ages, the mean MetHb percent in the control population is actually higher than that of the study population, but this is not statistically significant. However, on further analysis it was shown that 31.5% of the infants up to 60 days of age in the study

Table VIII-1. METHEMOGLOBIN IN INFANTS IN AREAS WITH HIGH AND LOW NITRATE CONCENTRATIONS IN DRINKING WATER

	Mean NO_3 Mg/l	1-60 days n	1-60 days MetHb%	61-90 days n	61-90 days MetHb%	91+ days n	91+ days MetHb%	All ages n	All ages MetHb%	S.D.
Control area	5	96	1.30	75	1.24	556	.97	728*	1.11	.72
Study area	50-90	71	1.39	188	1.14	1426	.99	1702*	1.01	.72
Total		167	1.33	263	1.17	1995	.98	2473*	1.04	.72

* Total includes infants with age unknown.

Table VIII-2. DISTRIBUTION OF HEMOGLOBIN LEVELS AMONG INFANTS

(Age, 1-18 months)

Hb (g%)	Jerusalem Jews	Jerusalem Arabs	Nes-Ziona	Rehovoth	Rishion Le Zion
up to 9.0	1.5%	5.5%	4.1%	1.0%	0.9%
9.1 - 10.0	8.6	13.1	9.3	6.5	5.6
10.1 - 11.0	21.9	28.0	26.9	18.1	18.3
11.1 - 12.0	29.7	28.0	29.2	33.5	37.6
12.1 - 13.0	21.5	16.9	19.0	13.0	25.3
13.1 - 14.0	10.1	3.4	5.4	13.0	8.2
14.1 +	4.3	3.7	2.9	2.1	3.8
unknowns	3.6	1.3	3.1	0.7	0.4
numbers of infants	606	236	484	828	466
Hb mean (g%)	11.6	11.2	11.4	11.5	11.8

population have MetHb levels higher than 1.9% as against 11.9% for the controls. Table VIII-3 shows the hemoglobin levels of the groups studied. The means of each group are almost identical.

Only 5% of the infants studied received powdered milk formula made up with tap water. The remaining 94% of the infants were either breast-fed and/or received whole cow's milk. Those infants fed with powdered milk formula in the study area showed somewhat higher MetHb percent than those fed exclusively on other types of milk (see Table VIII-5). There are only 35 infants in this category and the differences are not significant. Detailed analysis of the effect of age, vitamin C intake, diarrhea on MetHb levels did not reveal any significant differences in this group as compared to the control.

Forty-nine percent of the infants in the 1-60 day age group consumed either citrus or tomato juice or both. For the 61-90 days group, 80%, and over 91 days, 91% consumed such vitamin C rich foods. The mean consumption for the total population studied was 87%. In Table VIII-4 the MetHb levels in infants with or without vitamin C rich foods in their diet are presented. The 1-90 day age group of the study population consuming citrus or tomato juice show somewhat lower MetHb levels as compared to the non-consumers. In the control group and in those over 91 days in age there were no differences.

In the 1-90 day age group infants reported to be suffering from diarrhea on the day of examination or within the last month showed higher MetHb in both the study and control area (see Table VIII-6). In the study area, 1-90 day infants with diarrhea had a mean of 1.78% MetHb as compared to 1.16% in infants not suffering. Infants of 1-90 days with diarrhea from the study area showed higher MetHb than those from the control area. The numbers in this category are small and the differences are not significant.

There were essentially no differences among infants above 91 days in age.

MetHb is significantly higher in the first 60 days of life in both study and control populations (Table VIII-1). Table VIII-6 shows that the mean for both groups is 1.33% for the 1-60 day group, and 1.17% for the 61-90 day group. Infants over 90 days show MetHb levels of about 1%, with the mean of 1.04% for the total population studied. The same patterns are found with present weight as shown in Table VIII-7.

DISCUSSION

The fact that there are no significant differences between the mean MetHb levels in the 1,720 infants in the study area with a mean nitrate concentration of 70 mg/l in the drinking water as compared to the 756 infants in the control area does not support the findings of substantial

Table VIII-3. METHEMOGLOBIN IN INFANTS DRINKING POWDERED MILK FORMULA AND ONLY OTHER FORMS OF MILK

	Powdered Milk		Only other Forms of Milk	
	n	Met.Hb%	n	Met.Hb%
Control area	111	.98	684	1.14
Study area	36	1.17	1626	1.00
Total	147	1.01	2310	1.04

Table VIII-4. METHEMOGLOBIN IN INFANTS WITH AND WITHOUT CITRUS OR TOMATO JUICE IN DIET

	Age 1-90 days				Age 91+ days			
	with		without		with		without	
	n	Met.Hb%	n	Met.Hb%	n	Met.Hb%	n	Met.Hb%
Control area	65	1.27	106	1.27	452	1.03	104	1.08
Study area	226	1.19	53	1.10	1366	.97	73	.98
Total	291	1.21	159	1.20	1818	.98	177	1.04

Table VIII-5. METHEMOGLOBIN IN INFANTS WITH AND WITHOUT DIARRHEA

	Age 1-90 days				Age 91+ days			
	with		without		with		without	
	n	Met.Hb%	n	Met.Hb%	n	Met.Hb%	n	Met.Hb%
Control area	55	1.43	157	1.23	186	.99	369	1.06
Study area	20	1.78	356	1.16	165	1.01	1254	.96
Total	55	1.56	376	1.18	353	1.00	1635	.98

Table VIII-6. METHEMOGLOBIN AS A FUNCTION OF AGE

Age in Days	N	Mean(%)
1 - 60	187	1.35
61 - 90	363	1.17
91 - 120	335	1.07
121 - 180	573	.99
181 - 270	461	1.00
271 - 360	411	.94
361 - 450	277	.99
451 - 540	161	.97
541+	57	.85
Unknown	49	-
All Ages	2473	1.04

Table VIII-7. METHEMOGLOBIN BY PREMATURE WEIGHT

Wgt in Kgs.	N	Mean(%)
0 - 4.0	38	1.37
4.1 - 5.0	204	1.30
5.1 - 6.0	303	1.15
6.1 - 7.0	341	1.08
7.1 - 8.0	408	.99
8.1 - 9.0	431	.95
9.1 - 10.0	317	.95
10.1 - 12.0	375	.90
12 -	58	.97
Unknown	70	-
Total	2357	1.04

methemoglobinemia reported by Knotek and Schmidt(7) in Czechoslovakia. A possible explanation for this lies in the differences in infant feeding practices found in Israel where only 6% of the infants included in the study received appreciable amounts of tap water together with formula prepared from powdered milk. Measurements of fluid intake in infants 1-3 months of age indicate that during hot dry periods their intake of tap water given as a supplement is about 55%-60% of their total daily liquid intake, which is mainly made up of mothers' milk or cows' milk. However, there were no indications of higher Metth levels during the summer months despite somewhat higher intake of tap water. The specific role that powdered milk per se may play in raised Metth levels as suggested by Knotek and Schmidt(8) is still a moot point. Another factor may be the widely practiced feeding of vitamin C rich foods such as citrus and tomato juice to infants. Both foods are inexpensive and available during most of the year in Israel. Eighty-seven percent of the infant population studied consumed such vitamin C rich foods which are known to be an effective antidote to methemoglobinemia. In Central Europe it can be assumed that such vitamin C rich food supplements are expensive and not always in supply so that they are much less frequently used.

The finding that infants in the study area receiving powdered milk formula or no vitamin C rich food supplements had slightly higher Metth than their controls is supportive of this hypothesis, although the number of infants in each category was small and the differences are not statistically significant.

The higher Metth levels in infants (1-90 days) reported to be suffering from diarrhea in both the study and control area supports clinical findings in Israel of an association between infant diarrhea and methemoglobinemia(9). The fact that in the study area the level of Metth in infants with diarrhea was higher than similar infants in the control area may be suggestive of an additive effect resulting from the higher exposure to nitrates in water. But there again the number of infants in these categories is small and the differences not statistically significant.

The finding of higher Metth levels in the first 90 days of life in both study and control groups supports similar findings by Shearer et al(9).

One conclusion from this study is that no apparent public health problem associated with infant methemoglobinemia was detected in the study area, despite the fact that most of the wells were supplying water with nitrate concentrations above that generally recommended by public health authorities. However, it would be incautious to extrapolate from these findings as to the situation that may exist in other areas where infants consume appreciable amounts of tap water together with milk formula and where vitamin C rich foods are not widely given as diet supplements to such young infants(10).

SUMMARY AND CONCLUSIONS

Two thousand four hundred seventy-three infants in the Rehovot area with medium-high (50-90 mg/l as NO_3) and in Jerusalem with low (5 mg/l as NO_3) concentrations of nitrate in drinking water were studied in an effort to determine whether there is any association between methemoglobin (MetHb) levels and nitrates in drinking water.

MetHb levels are highest in the first 60 days of life with a mean of 1.33%. The mean MetHb level for the entire infant population studies was 1.044.737.

No differences were found between the mean MetHb level in the study and control areas.

A possible explanation for this finding lies in the fact that only 6% of the infants consumed appreciable amounts of tap water together with powdered milk formula. The remainder were breast-fed or consumed whole cow's milk. Eighty-seven percent of the infants were fed vitamin C rich foods such as citrus or tomato juice which are known for their anti-MetHb effects. It cannot be assumed that there is no danger from nitrates in drinking water in areas where infants consume larger amounts of tap water with powdered milk formula and/or little vitamin C rich foods.

REFERENCES

1. Comly, H.H., Cyanosis in infants caused by nitrates in well water. J.A.M.A. 129, 112, 1945.

2. Gruener, N. and Shuval, H.I., Health aspects of nitrates in drinking water. In Developments in Water Quality Research (p 89-108), Ann Arbor Humphrey Science Publishers, Ann Arbor: London, 1970.

3. U.S. Public Health Service Drinking Water Standards, PHS Publ. No. 956, 1962.

4. Walton, G. Survey of the literature relating to infant methemoglobinemia due to nitrate contaminated water. A.J.P.H. 41:986, 1951.

5. Knotek, Z. and Schmidt, P. Pathogenesis, incidence and possibilities of preventing alimentary nitrate methemoglobinemia in infants. Pediatrics 34,76, 1964.

6. Evelyn, K.A. and Malloy, H.T. Microdetermination of oxyhemoglobin, methemoglobin and sulfhemoglobin in a single sample of blood. J. Biol. Chem., 106, 655, 1938.

7. Hegesh, E., Gruener, N., Cohen, S., Boukhovsky, R., and Shuval, H.I. A sensitive micromethod for the determination of methemoglobin in blood. Clin. Chem. Acta., 30:679-683, 1970.

8. Levine, S., Chief of Pediatrics, Kaplan Hospital, Rehovot, personal communications.

9. Shearer, L.A., Goldsmith, J.R., Young, C., Kearns, G.A., and Tompkin, R. Methemoglobin levels in infants in an area with high nitrate water supply. Am. J. Pub. Health 62:1174-460 (1972).

10. Shuval, H.I., and Gruener, N. Epidemiological and toxicological aspects of nitrates and nitrites in the environment. Am. J. Pub. Health 62:1045-1052. (1972).

SECTION IX

EPIDEMIOLOGICAL STUDY OF THE ASSOCIATION BETWEEN NITRATES IN DRINKING WATER AND METHEMOGLOBIN LEVELS IN INFANTS

GAZA AREA

INTRODUCTION

On completion of the epidemiological study in the Rehovot area it became apparent that very few infants in that area were actually exposed to nitrates in drinking water in the form of powdered milk formula since nutritional regime is rarely practiced in Israel today.

Children receiving only cow' milk or breast fed did however receive tap water supplements amounting to about 10% of their total liquid intake in winter and as high as 50% in summer.

Previous studies indicate however that only infants exposed to appreciably quantities of high nitrate tap water made up as powdered milk formula could be considered as candidates for raised Meith levels. It is still a mute question as to the role of the powdered milk per se in the etiology of methemoglobinemia. In order to study the possible association between nitrates in drinking water consumed in powdered milk formula and raised Meith levels in infants, an area where this type of infant feeding is practiced had to be found.

The Gaza area was considered to be favorable for such a study since preliminary investigations indicated that well over 50% of the infants up to two years of age received powdered milk formula with tap water. In addition there were many local areas in Gaza with high nitrate concentrations in the drinking water. A survey of the local wells indicated that many of them had as much as 135 mg/l of nitrates as NO_3 or three times that of the recommended maximum concentration.

METHODS

In 1972 and 1973 a field study was mounted in the Gaza region with the full cooperation of the local public health services, after numerous administrative difficulties which prevented an earlier start had been overcome.

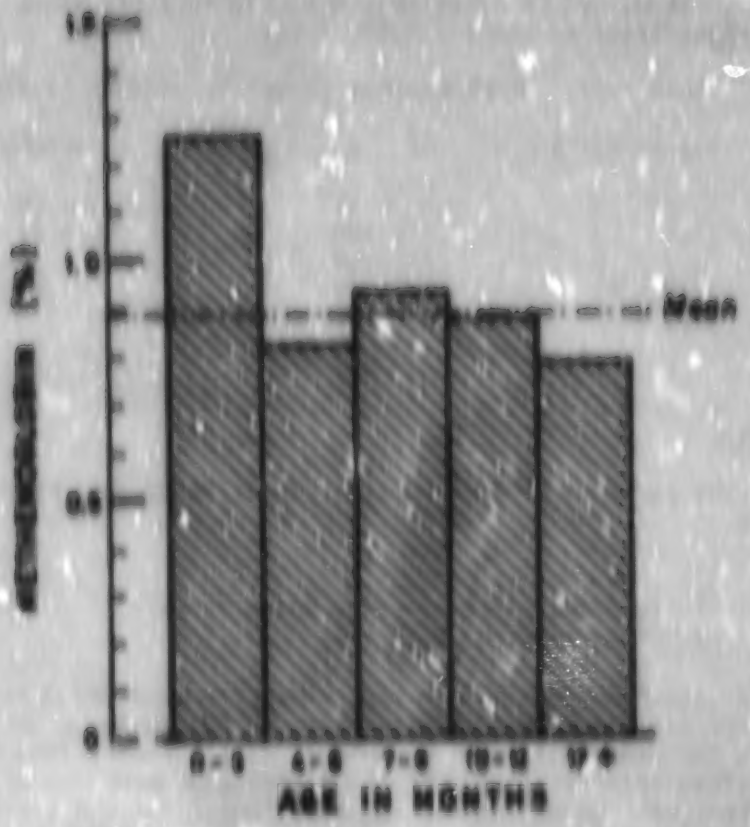


Figure IX-1. METHEMOGLOBIN LEVELS IN INFANTS BY AGE GAZA

RESULTS

Age

As had been found in our previous study the MetHb level in the first three months of life was higher than after that period regardless of exposure to nitrates, as can be seen in Table IX-1 and Figure IX-1. The MetHb level for the 45 infants three months of age or less is 1.20% as compared to about .6-.9% after that age. These differences are statistically significant at the p = 0.005 level.

Table IX-1. METHEMOGLOBIN LEVELS IN INFANTS BY AGE

Age in Months	N	MetHb%
0 - 3	45	1.20
4 - 6	106	0.61
7 - 9	109	.92
10 - 12	67	.68
13 +	91	.79
All	418	.85

Milk regime

For the purposes of this analyses three categories of milk regimes were used.

a) Powdered milk only.
b) Powdered milk and other forms of milk.
c) Other forms of milk only.

It is assumed that infants consuming only powdered milk would have the highest nitrate intake from tap water while those on the mixed powdered milk and other milk regime would receive somewhat less tap water and therefore less nitrates. Those receiving no powdered milk would consume the least amount of tap water and would not therefore be exposed to nitrates if present in the water.

Table IX-2 (total) column 1-3) shows that infants on "powdered milk only" regime have higher MetHb levels than those on the mixed regime, while the latter show higher MetHb levels than those consuming no powdered milk. However, a fuller analysis requires looking at each milk regime category in terms of the potential nitrate exposure from tap water in each individual case (see next paragraph).

Table IX-2. MEAN HYDROGLOBIN LEVELS IN INFANTS ON DIFFERENT MILK

FEEDING CONSISTING WATER OF VARYING NITRATE CONCENTRATIONS

	Gm									
	1 Low Nitrates		2 Medium Nitrates		3 High Nitrates		2 + 3 Medium + High Nitrates		1 - 3 Total	
Milk Regime	n	Metab	n	Metab	n	Metab	n	Metab	n	Metab
a) Powdered milk only	7	.69 *(.25)	32	.74 (.55)	22	1.37 (2.80)	54	1.18 (2.27)	4[illegible]	1.12 (2.05)
b) Powdered plus other	90	.75 (.46)	58	1.08 (1.26)	124	.94 (1.08)	182	.99 (1.14)	27[illegible]	.90 (.97)
a+b) Powdered milk only or some	97	.75 (.45)	70	1.04 (1.17)	146	1.01 (1.47)	216	1.02 (1.37)	313	.95 (1.16)
c) No powdered milk	26	.79 (.80)	15	.58 (.28)	54	.65 (.55)	69	.77 (.51)	95	.78 (.60)
a-c All	123	.74 (.54)	85	.96 (1.08)	200	.96 (1.26)	285	.96 (1.23)	** 408	.89 (1.06)

* Number in ( ) is standard deviation
** Includes 19 cases of nitrates unknown

Low nitrates – 44 mg/l NO_3 or less (mean = 32.5 mg/l)
Medium nitrates – 45-55 mg/l NO_3 (mean = 50 mg/l)
High nitrates – 56 mg/l NO_3 and greater (mean = 87 mg/l)

Nitrates in drinking water

For the purposes of this analysis three nitrate concentration categories were established:

1. Low nitrates: 44 mg/l NO_3 or less (mean = 32.5 mg/l)
2. Medium nitrates: 45-55 mg/l NO_3 (mean = 50 mg/l)
3. High nitrates: 56 mg/l NO_3 and greater (mean 67 mg/l)

The low nitrate category represents water within the limits of the current recommended maximum for drinking water, i.e. 45 mg/l NO_3. The mean given is the actual mean nitrate level in the drinking water of the 123 cases examined in this category. The medium nitrates category represents the border area immediately above the present standard, while the high nitrates category includes all 200 cases above 56 mg/l NO_3, with a mean twice that of the standard. In that category 58 cases were exposed to nitrate concentrations greater than 80 mg/l with a mean of 131 mg/l.

As can be seen from Table IX-1 and Figure IX-2, there are essentially no differences in MetHb levels among infants in the low nitrate category regardless of the milk regime. The mean MetHb for the low nitrate group being .74%, the small differences seen are not significant statistically.

In the medium nitrate group there appears to be a raised level of MetHb in the two groups consuming powdered milk as compared to the group consuming only "other milk" in the medium nitrate category and as compared to the low nitrate group.

The trend that is first indicated in the medium nitrate group becomes clearer in the high nitrate group with those infants consuming "powdered milk only" showing a raised MetHb level of 1.57%, almost twice that of the low nitrate group. Here it also appears that those consuming only powdered milk show higher MetHb levels than those on the mixed regime, while those on the mixed regime have higher MetHb levels than infants consuming only "other" forms of milk.

For the purposes of statistical analysis it was found necessary, however, to combine the groups consuming only powdered milk (a) with that consuming powdered milk as well as other forms of milk (b) so that the numbers of infants in each category would be larger. This was considered feasible since there were no statistically significant differences between the two groups. Statistical tests show that infants consuming some amount of powdered milk made up with water containing high concentrations of nitrates (3 a+b) had significantly raised MetHb levels as compared to those in the low nitrate area regardless of the type of milk consumed (1 a-c) with p= 0.035.

There were similar differences in the medium nitrate group. Those consuming "powdered milk" (2 a+b) were compared to all infants in the low nitrate group (1 a-c) with p = 0.04.

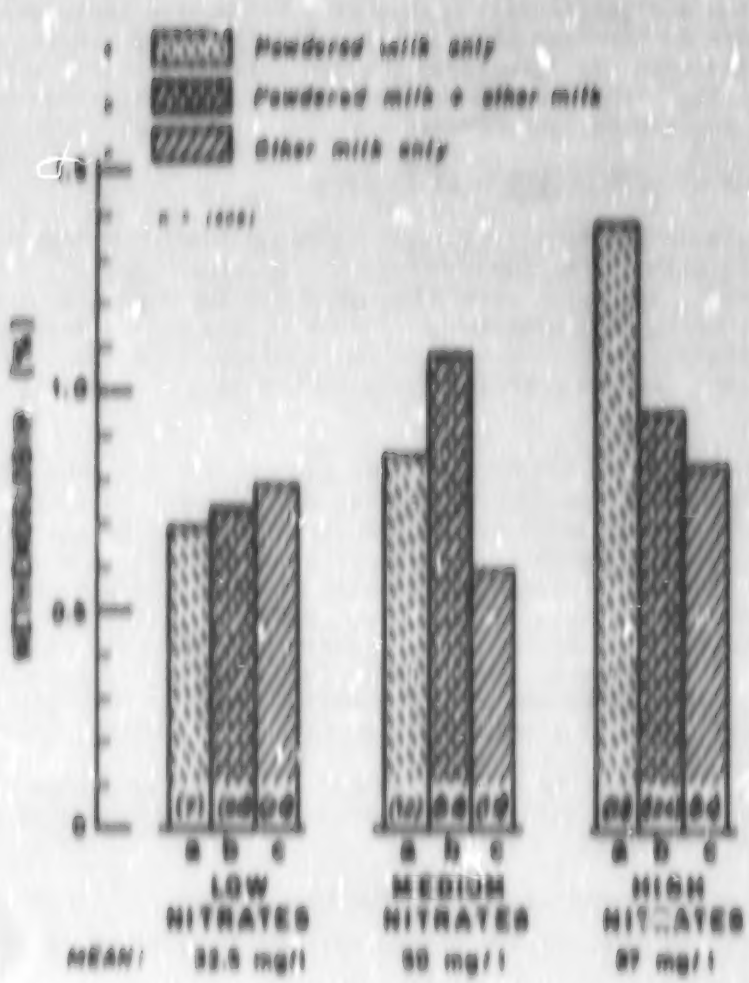


FIGURE 18-2. MEAN METHEMOGLOBIN LEVELS IN INFANTS ON DIFFERENT MILK REGIMES, CONSUMING OF LOW AND MEDIUM TO HIGH CONCENTRATIONS OF NITRATES - GAZA.

The infants in the medium and high nitrate group were also pooled for further statistical analysis since there were no statistical differences between these two groups.

Infants consuming powdered milk in the medium-high nitrate group (2+3-a+b) had a significantly higher mean MetHb than those consuming no powdered milk in the same nitrate group 2+3 c) with p = 0.05; as well as when compared with all infants in the low nitrate group (1 a-c) with p = 0.005. The high level of significance in the difference between these last two groups is noteworthy.

DISTRIBUTION OF METHEMOGLOBIN IN INFANTS

An analysis of the distribution of MetHb levels of infants was made according to milk regime and exposure to nitrates. For the low nitrate group all milk categories were combined while for medium-high nitrate group all infants consuming powdered milk to any extent were combined. The low nitrate category is the same as in Table IX-3 while medium and high nitrate groups were pooled since both groups showed raised MetHb levels.

The MetHb levels are divided into two categories. The first 0-1.7% and the second 1.8% and over. This second group was selected on the assumption that 1.8% MetHb or over represents significantly raised levels of MetHb. The mean MetHb levels found in all infants in the low nitrate group was .74% with a standard deviation of .54%. It can be assumed that infants with MetHb levels of 1.8 or greater (i.e., the mean plus 2 S.D.) can be categorized as having significantly raised levels of MetHb. From Table IX-5 it can be seen that 3.2% of the infants in the low nitrate group show raised MetHb while in the medium and high nitrates group 10.7% of the infants consuming powdered milk have raised MetHb levels.

Only 4.6 of those in the medium and high nitrate groups consuming no powdered milk show raised MetHb levels. These differences are significant at the 0.05 level.

Table IX-5 DISTRIBUTION OF METHEMOGLOBIN LEVELS IN INFANTS EXPOSED TO HIGH AND LOW CONCENTRATIONS OF NITRATES IN DRINKING WATER - GAZA

	Milk Regime	0-1.7% MetHb n	%	1.8%+ MetHb n	%	Total n	%
Low Nitrates	All	117	96.8	4	3.2	121	100
Medium + High Nitrates	Powdered Milk	193	89.3	23	10.7	216	100
	No powdered Milk	65	95.4	3	4.6	68	100
	All	259	90.9	26	9.1	285	100

OTHER VARIABLES

Information was gathered for each infant concerning diet, in particular the consumption of high nitrate foods such as spinach and foods rich in ascorbic acid which is an antidote for methemoglobinemia. No significant findings evolved from an analysis of such dietary variables.

A further analysis controlling for age of the infants could not provide significant findings.

In another phase of this project we were able to show that tap water intake is higher in the hot summer months. An analysis of MetHb levels by month of the year did not however provide any significant findings.

As an additional control group, 105 infants were tested for MetHb levels in Jerusalem where nitrate levels in the water supply are very low. The mean MetHb found was .79% which is similar to the MetHb of infants in low nitrate areas in Gaza - .74%.

It is noted that the mean MetHb levels of the low nitrate group in Gaza and the control group in Jerusalem are lower than the means found in the Rehovot area study. These slightly lower MetHb levels are a result of refinements in the test method which developed in the period since the original study was initiated in 1970 and 1971.

DISCUSSION

The Gaza study has provided some further confirmation that infants in areas with water supplies having concentrations over 45 mg/l of nitrates (as NO_3) who consume appreciable amounts of tap water in powdered milk formula show raised MetHb levels. Although no clinical cases of infant methemoglobinemia were revealed among 205 infants in the medium and high nitrate areas, 26 of them showed significantly raised MetHb levels of over 1.5%, of them 23 (10.7%) recieved either only powdered milk formula or powdered milk formula in addition to other types of milk. Only 3.3% of the infants in the low nitrate group showed raised MetHb.

Infants in the low nitrate group show similar MetHb levels regardless of milk regime while infants who consume powdered milk in the medium nitrate group with a mean nitrate concentration of 50 mg/l in the water have a significantly raised mean MetHb level. This difference appears more clearly in the high nitrate group. It is worthy to note that the mean MetHb level of the 22 infants on "milk powder only" and who received water having high nitrate concentrations (mean 87 mg/l of NO_3) had a mean MetHb level of 1.37% as compared with a mean of 1.30% for the 104 hospitalized infants exposed to approximately the same level of nitrates in their powdered milk formula reported upon elsewhere in this report.

The possibility that infants in the medium and high nitrate areas consuming powdered milk were younger than the population studied and

therefore normally might be expected to have higher MetHb levels was examined. Of the infants in the medium and high nitrate area 10.6% were three months or younger and 66% were six months or younger as compared with 10.8% and 50% for the total sample studied. Thus, there is no indication that the higher MetHb levels in the infants consuming powdered milk mixed with medium or high nitrate water could be explained by age differences.

It was impossible in this study to clarify the role of milk powder per se in conjunction with high nitrate water in the etiology of raised MetHb levels. Undoubtedly infants on milk powder regimes consumed the highest amounts of high nitrate tap water. Our studies on the consumption of drinking water by infants do indicate that during the hot summer months as much as 50% of the total liquid intake could be in the form of supplemental tap water. In low nitrate areas, milk regime had no influence in MetHb levels, however.

The Gaza study appears to provide support for the present maximum recommended standard of 45 mg/l of NO_3 in drinking water. The fact that the first signs of raised MetHb levels clearly appear in infants exposed to water just above the standard (45-55 mg/l NO_3) suggests that little if any safety factor is provided by this standard.

The full health significance of slightly raised MetHb levels such as reported upon here is yet to be established. Whether such sub-clinical methemoglobinemia is deliterious in itself or whether such exposure is only of importance to the extent that clinical cases of the disease develop requires further study.

The question of why and how subclinical methemoglobinemia develops in certain individuals into the clinical form of the disease still remains unanswered. Other possible direct toxic effects of nitrates and nitrites cannot be overlooked as well. These will be discussed elsewhere in this report.

In conclusion, we must point out that the association between the exposure to nitrates in drinking water in the form of powdered milk formula and raised MetHb levels in infants has been demonstrated even though certain inconsistencies associated with a field study of this type are apparent.

Even water containing nitrates slightly above the current standard is not free from suspicion. Such findings even in the absence of clear-cut clinical cases of the disease must be considered as further evidence supporting the current recommended standard of 45 mg/l, in those areas where infants consume appreciable amounts of tap water in the form of powdered milk formula. One might even question the degree of protection provided by that standard when large population groups are involved since there is evidence from this study that little, if any, safety factor is provided by it.

SECTION 5

THE EFFECT OF CHANGES IN NITRATE CONCENTRATION IN DRINKING WATER ON METHAEMOGLOBIN LEVELS IN INFANTS
A CONTROLLED HOSPITAL STUDY

Under normal field conditions many difficulties hamper the possibility of detecting a dose-response relationship between nitrate intake and methaemoglobin levels especially in the low range. To overcome some of these problems, a controlled experiment was carried out in a hospital. In this study, we attempted to determine the threshold value of nitrates in water which can cause a significant increase above "normal" methaemoglobin (MetHb) levels in infants.

METHODS

A controlled experiment was carried out in the pediatrics ward of the Hillel Joffe Hospital, Hadera. In the course of the national survey it was revealed that this hospital is normally supplied with high nitrate content water. Patients, however, come from both low and high nitrate areas. For five days, 104 infants ranging from one week to ten months were exposed exclusively to water whose nitrate content was exactly controlled. As was the normal practice in the ward, the infants received mainly formula prepared from milk powder, a fact which increased their water intake as compared with fresh whole milk feeding which is the routine diet regimen in Israel.

The exposure schedule was as follows:

1. First day: low nitrate content (mean 15 mg/l)
2. Second day: High nitrate content (mean 108 mg/l)
3. Third day: High nitrate content (mean 108 mg/l)
4. Fourth day: High nitrate content (mean 108 mg/l)
5. Fifth day: Low nitrate content (mean 15 mg/l)

The high nitrate water was from the local well which normally supplied water to the hospital, and the low nitrate water was specially brought from a distant one. Water was kept in special containers and the infants were supplied solely from this source. MetHb levels in infants were measured by a sensitive method previously reported(1). Finger blood samples were taken in the morning after the infants had been exposed to the specific water for 24 hours. Each infant was checked and followed individually. The results were statistically analysed according to Wilcoxon(2). It should be noted here that the District Public Health Office had recommended that the hospital alter its water supply even before this study was initiated, and this has meanwhile been done.

RESULTS

The infant population of 104 consisted of 56% females and 44% males. Three quarters were hospitalized because of intestinal problems and one-third of them received infusions to provide extra fluid intake. Exact

Table X-1. METHEMOGLOBIN LEVELS IN INFANTS EXPOSED TO HIGH AND LOW NITRATES IN WATER

Day	Nitrate	No. of Infants	Mean of Metgb in percent
1	low	87	0.89
2	high	93	1.30
3	high	85	0.81
4	high	83	0.93
5	low	75	0.80

Table X-2. DISTRIBUTION OF CHANGES IN METHEMOGLOBIN LEVELS BETWEEN DAYS OF VARYING EXPOSURE TO NITRATES IN WATER

Compared	Number of Infants	d = 0 n	%	d+ n	%	d- n	%	P increase	P decrease
1 - 2	79	4	5	46	59	29	37	0.02	-
2 - 3	80	8	9	35	44	38	48	-	0.24
3 - 4	72	8	11	28	37	36	52	-	0.14
4 - 5	59	5	8	68	18	36	61	-	0.02
1 - 5	66	10	15	22	33	34	51	-	0.26

records of the water intake could not be taken. Table X-1 represents the means of MetHb levels during the five days of the experiment. The first stage of the low level nitrate intake was intended to reveal the individual level each infant would show when little nitrate was included in its diet. It is suggested that only the addition to this baseline level should be ascribed to the exposure to nitrates on the second, third and fourth day. The additional low level of nitrate of the fifth day was intended to reveal in what degree MetHb levels in the infants would react to the cessation of nitrate exposure.

There was a significant rise in the mean MetHb levels in the second day compared to the first one, i.e., following the first exposure to nitrate. There were three cases of massive rise in MetHb levels, all between the first and second day. The rises amounted to 5.5%, 10.4%, and 14.5%, reaching levels of 6.9%, 11.9%, and 15.9%. The mean MetHb level on the third day decreased almost to the original level in spite of the fact that the high exposure continued. It remained constant on the fourth day (high nitrate intake) but dropped even lower than the first day on the fifth day (low nitrate again).

The Wilcoxon test was used to analyse whether the shift in MetHb levels for the series of individual infants on days of varying exposure to nitrates in drinking water was truly significant. Examining the difference between the means of each day might lead to misleading results since a few infants showed relatively large changes in MetHb levels. By this method each child is scored according to its value relatively to the other observations. In Table X-2 this analysis is presented and it can be seen for example that between day one and two 50% of the infants showed an increase in MetHb levels (d+) as compared to only 37% showing a decrease (d-). The increase in MetHb levels between days one and two, resulting from the first exposure to nitrates was significant. The decrease in MetHb levels between days four and five after the infants were once again supplied to low nitrate water was also significant.

The above observations were made on 104 infants even though not all infants were tested every day since some left the hospital and others were missed for various technical reasons. There were 57 infants whose blood was taken on each of the five days. The findings of these 57 infants reveal the same tendencies as the whole population, though in a more extreme pattern.

DISCUSSION

Epidemiological field surveys of methemoglobinemia induced by nitrates in drinking water face a number of difficulties: 1) Nitrate levels in the water vary over a wide range even at a single source(5). 2) Excessive boiling of water to be used for powdered milk formula can increase the nitrate concentration. 3) Measurement of methemoglobin levels at a short interval after nitrate intake is generally not feasible. This last point is particularly important since elevated MetHb induced by nitrates returns

to normal relatively quickly. It has been shown that in each 00 min. period the level drops to 50%(4). We were able to control at least these three variables in this study carried out in a hospital pediatrics ward.

The results show that although the nitrate level in the water was more than double the recommended standard for drinking water and the infants drank the water for three consecutive days, essentially no clinical methemoglobinemia developed. Most of the infants reacted to the increase in the nitrate level between the first and second days by a parallel increase in MetHb level. In spite of the continued high intake of nitrates on the third and fourth days, there was an apparent drop in the mean MetHb level on the third and fourth days. More cases showed a fall in MetHb than an increase between the third and the fourth days. Although these changes could not be shown to be statistically significant which may be due to the large variability and the small sample, it may be a hint at a mechanism of adaptation which reacts quite rapidly to increased exposure of nitrates. There is a possibility that such a mechanism may be already developed among infants coming from high nitrate areas and this connects the problem to much more complex question.

The day after the exposure to high nitrates in water stopped, MetHb levels fell to the initial pre-exposure level. This fact demonstrates the rather fast reactions of MetHb levels to changes in nitrate levels in the diet. While previous reports indicate that several weeks of exposure to high nitrate water is required before raised MetHb levels are detected, our findings indicate that at least under certain conditions, such long "incubation periods" do not necessarily apply. The prevalence of gastroenteritis among the hospitalized infants might explain their predisposition to developing high levels of MetHb after one day of exposure to high nitrate water.

This work indicates that nitrate levels in drinking water of about 100 mg/l can cause a significant increase in infant MetHb levels. If this exposure is stopped recovery is rapid. Despite the fact that no clinical methemoglobinemia developed among the 104 infants studied, the question as to the possible long-term effects resulting from continuous exposure to elevated nitrate levels in water used in infant formula has not yet been determined. The possibility of an adaptation mechanism remains to be elucidated as well.

REFERENCES

1. Hagean, R., Gruener, N., Cohen, G., Bochkovsky, R., Shuval, H.I. A Sensitive micromethod for the determination of methemoglobin in blood. Clin. Chim. Acta. 30:670-685, 1970.

2. Siegel, S. Non-parametric Statistics, McGraw-Hill Book Company, Inc., New York, p 75-83, 1956.

3. Saliternik, C. Ground water pollution by nitrogen compounds in Israel, in Advances of Water Pollution Research, Ed. S. H. Jenkins, Pergamon Press, London, p 171-179, 1973.

4. Gruener, N. and Shuval, H.I., in Environmental Quality and Safety, Vol. II (ed. F. Coulston and F. Rosto), Georg Thium Verlag, Stuttgart, and Academic Press, New York, p 219, 1972.

SECTION XI

SURVEY OF LIQUIDS INTAKE IN INFANTS

Limited information exists in the literature concerning water intake in infants. Such information may be of importance for a variety of reasons: 1) Evaluation of the risks to infants and children from exposure to harmful chemicals in water. 2) Intake of nutritional constituents in water which may be of health importance. 3) The physiological importance of sufficient water intake particularly in hot areas.

One of the main hypothesis in our previous study which indicated no difference in methemoglobin levels between the study area (high nitrate in water) and control areas was that in both cases there is essentially low exposure to nitrates from drinking water due to the normal dietary practices in Israel today. As was reported, 96% of the infants included in the Rehovot area study were either breast-fed or received whole cow's milk as their main source of liquid intake. The use of powdered milk formula has practically disappeared in the Jewish population of Israel. Tap water is given to such infants as "tea" or sugar water or plain tap water as a supplement only. No reliable information was available from pediatricians, or from pediatric departments at several hospitals as to the amount of supplemental water usually given to infants in the 1-6 months age group. Therefore it was difficult to evaluate the contribution of supplemental water as a nitrate source for infants whose main liquid source contains no tap water. To answer this question we initiated a survey of water intake among infants in the Rehovot-Rishon-le Zion area (high in nitrate).

METHOD

A liquid intake survey was carried out directly with the mothers under the supervision of our staff nurse. We designed a detailed record book with one page for each day of the month. The page was divided to ease the recording of the information for different liquid and food types and also by hours of the day - three sections and one section for the night. Before starting the study the mother was interviewed by a staff nurse and was instructed in the use of the record book.

Each week, each mother was visited by the nurse who inspected the record book and advised on any problems that may have arisen. The daily amounts of liquid intake were recorded differentiating between milk and different "types" of tap water. Other sources of liquids were negligible. One hundred fifteen infants were included in the survey between the ages of one to five months, who were followed for one month periods. The pre-test was made in January 1972 and then the study continued for a 12-month period starting June 1972.

RESULTS AND DISCUSSION

There is a marked decrease in the unit liquid intake (ml/kg of body weight/day) with age (Table XI-1).

Table XI-1 LIQUID INTAKE IN INFANTS
AGE 1 TO 5 MONTHS
ml/kg/day

Age in Months	1	2	3	4	5
Mean intake	184.6	135.50	102.10	86.9	69.2
n	34	37	33	8	13

This decrease parallels the increase in the body weight of the infants. A relationship between water intake and the monthly average ambient temperature (Figure XI-1) was noted, with the highest water intake occurring during the hottest months (July and August) and with the lowest in cold season. The correlation between age and the specific liquid intake was found to be significant with a correlation constant of 0.65. Exactly the same figure was found between liquid intake and body weight.

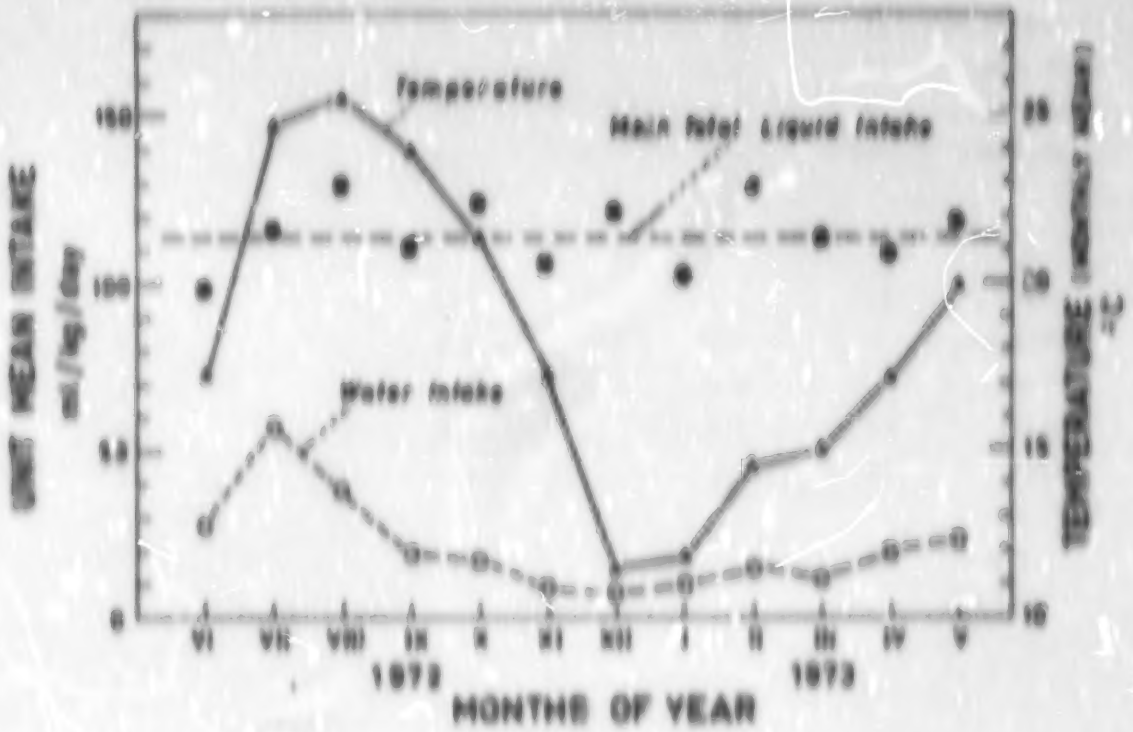


Figure XI-1. TOTAL LIQUID AND WATER INTAKE IN INFANTS 1-5 MONTHS OLD, ACCORDING TO THE MONTHS OF THE YEAR.

Table 31-8. LIQUID INTAKE AND TAP WATER INTAKE
IN INFANT ACCORDING TO MONTH OF YEAR

Month	n	Average age Month	mean liquid intake ml/kg/day	mean water intake ml/kg/day
1977				
January	10	2.5	117	12
June	12	3.0	96	27
July	7	2.6	116	57
August	5	2.4	129	38
September	12	2.5	110	19
October	19	2.4	122	17
November	7	2.7	106	9
December	6	3.1	121	7
1978				
January	10	2.5	102	10
February	5	2.7	128	14
March	6	3.0	115	11
April	10	2.5	108	19
May	9	3.2	119	25
Mean			113	

The total liquid intake did not change and remained constant throughout the year. Analysis of the results in relation to ethnic origin showed a small difference between Ashkenazim and Sepharadim infants which may be attributed to cultural factors. Only two infants were fed with powdered milk formula (both now in Israel coming recently from the U.S.A.). As can be seen from Table XI-4 their liquid intake was similar to the majority of the infants who were fed on cow's milk. A minority was breast fed, got an extra liquid supply which amounted to a little higher than half of the intake of bottle fed infants of the same age group. The extreme cases recorded for the mean liquid intake ml/kg/daily were as high as 192 and as low as 52. The highest mean water intake was 65 and a baby with no water supplument was also recorded. The two infants fed on powdered milk (almost only water as liquid intake) had mean liquid intake of 109 and 145 ml/kg/day, respectively.

Table XI-3 LIQUID INTAKE IN INFANTS ACCORDING TO ETHNIC GROUP

ML/KG/DAY

	n	Mean intake
Ashkenazim	54	119
Sepharadim	46	102
Mixed	15	121

Table XI-4 LIQUID INTAKE IN INFANTS ACCORDING TO NUTRITIONAL REGIME

ML/KG/DAY

	n	liquid intake
Breast fed*	27	65
Pasteurized milk	84	118
Powdered milk	2	127

*only supplemental liquid intake recorded

The unit liquid intake in the first months of life is higher than during any other period of life. This is a physiological necessity and may be even more extreme in hot dry climates, as infants may suffer a severe loss of water and even in normal conditions infants insensible water losses are higher in the first year(1,2). Such high unit liquid intake may, however, endanger the child's health when the majority of the liquid is water which contains factors that may be harmful to his health.

Previous studies showed(3) that water intake in infants under one year of age were between 20-50% of total liquid intake. This is a little higher but not far from the results reported in this study. The water intake throughout the year can differ between the winter and the summer by a factor of 5. During the six cool months of the year the water intake amounted only to around 10% of the total liquid intake while during

the four hottest months reached 50% and up to 50% at the maximum. In colder climates such variations would not be expected as supplemental water would not be an important constituent of the diet in the first six months.

The observation that in spite of the large variations in water intake the total liquid intake remained constant was found also by Galagan et al(4) in their extensive study.

Such observation should be studied carefully in relation to its effects on the nutritional status of the infant during the hot seasons, since the remainder of the liquid intake is usually made up on milk which for many infants is the sole source of nutrition.

REFERENCES

1. Pratt, E.L., and Snyderman, S.E. Pediatrics 11, 65, 1955.

2. Heeley, A.M. and Talbot, N.B. Am. J. Dis. Child., 90, 251, 1955.

3. Margolis, F.J., Teate, H.L., Nuill, M.L., and Wilson, H.L. Water intake of normal children, Science, 140, 890, 1963.

4. Galagan, D.J., Vermillion, J.E., Hevitt, G.A., Stadt, E.M., and Dart, R.R. Climate and fluid intake, Pub. Health Report, U.S. 72, 484, 1957.

SECTION XII

ONTOGENETIC DEVELOPMENT OF NADH DEPENDENT METHEMOGLOBIN REDUCTASE IN ERYTHROCYTES OF DIFFERENT SPECIES

INTRODUCTION

NADH methemoglobin reductase (MR) has been suggested as the main reductive pathway of methemoglobin (MetHb) in the red blood cell(1,3). This assumption was based mainly on the *in vitro* relative activity in MetHb reduction. Correlation was also found between lower ability to reduce MetHb in young infants and their low MR activity(3,4). We have shown that the "normal" mean level of MetHb in infants up to three months old is significantly higher than the mean of 6-12 month old infants as well as that of adults(5).

Other studies in this laboratory carried out in different species show that mice, guinea pigs and rabbits, which have higher MR activity than rats, will show lower MetHb levels than rats when exposed to equal doses of sodium nitrite(6).

Epidemiological surveys reveal a relatively low prevalence of raised methemoglobin levels in infants exposed to nitrates in water. A number of factors have been indicated as playing a role in the predisposition to develop methemoglobinemia which include stomach pH, microflora in the upper intestine, liquid intake per unit of body weight and type of milk powder formula. All these are in addition, of course, to the amount of nitrates actually ingested. In addition to these factors we have hypothesized that sporadic clinical cases of methemoglobinemia in areas with high nitrate concentrations in water may arise from heterozygotes (in relation to MR deficiency). These cases generally can cope with the normal factors which convert Hb to MetHb but succumb when exposed to an abnormally large body burden of methemoglobinemia causing factor. Such individuals would start to show symptoms at lower nitrate dose levels than normal persons. Cases of both complete MR deficiency which cause methemoglobinemia, an autosomal recessive disease, and partial MR deficiency which has been shown to be susceptible to certain drugs, have been reported(7,8).

This study follows the development MR levels from the fetus to adult in both humans and rats, in anticipation that detailed information on the ontogenetic development of this vital enzyme in both species will be of value in understanding the pathogenesis of methemoglobinemia both under field conditions and in controlled laboratory studies.

METHOD

Ninety-nine cord-blood samples were taken from regular normal births. Finger blood samples were taken from 758 (438 Jews and 320 Arabs) infants appearing for routine check-ups at mother and child clinics. Ninety-nine adult blood samples were taken from blood bank donors. Heparin or EDTA were used as anticoagulants and found to have no effect, at concentrations used, on the enzyme activity. Samples were checked within 24 hours. Storage at 4°C for this length of time did not lead to changes in the enzyme activity. Zebra rats were used for the animal studies. Enzyme assay was done by a modification of Hugesh method(9).

RESULTS

Methemoglobin Reductase Activity in Man. The known phenomenon of lower activity in the newborn than adults has been confirmed. However, in testing numerous infants and children of different ages between birth and three years of age, a step-like pattern of MR activity increases appears to exist (Table XII-1). The rather low fetal level increased rapidly in the first days of life from 1.5 to 2.5 mumole/min/mg.Hb. This level remains for about 14 days. In the next step, 2 weeks to two months of age, there was an increase of 20%. Another increase of 15% brings the enzyme level at the age of 2-6 months to almost adult level. In the next step, 6-24 months, the enzyme level showed a plateau which is unexpectedly higher than adult level by 20% and drops down after two years to the adult level. An additional survey, which included 320 Arabs, showed a similar age profile with an increase to a plateau and a decrease after about two years. The MR activity in this population was lower compared to the Jewish infants and the plateau was shifted to slightly higher age.

Methemoglobin Reductase Activity in the Rat. While in humans the enzymatic level increases from the fetal to the adult level by 100%, in the rat the fetus has an MR activity which is about ten times higher than the adult (Table XII-2). This high level MR at birth decreases sharply in about two months, to the adult rat level. Some differences between males and females were observed in the younger age groups but this apparently disappears in the matured animals. Age profiles were followed also in the mouse and hen and the same trend as in the rat was found but in less extreme fashion.

DISCUSSION

Erythrocytic enzymes show different ontogenetic changes. In a survey done recently in human fetus erythrocytes(9) a number of enzymes such as hexokinase or Mg Atpase are higher in the fetus than in the newborn. Others, like NAD(H) MR and glutathione peroxidase, are lower during the intrauterine life than after birth. The reasons

Table XII-1. LEVELS OF METHEMOGLOBIN REDUCTASE ACTIVITY IN HUMANS AT DIFFERENT AGES

	m μ moles/min./mg.Hb					
	Jews			Arabs		
Age (Days)	n	mean	S.D.	n	mean	S.D.
Cord Blood	69	1.58	0.54	–	–	–
0 – 7	85	2.30	1.25	–	–	–
8 – 14	9	2.31	1.29	–	–	–
15 – 30	17	2.79	0.21	–	–	–
31 – 45	13	2.68	0.98	5	2.18	0.86
46 – 60	22	2.79	1.35	5	2.24	0.92
61 – 75	18	3.14	1.62	7	2.42	0.85
76 – 90	19	3.41	1.62	8	2.98	1.84
91 – 120	34	3.18	1.47	27	2.92	1.32
121 – 150	28	3.16	1.11	25	2.66	1.18
151 – 180	30	3.24	1.58	36	2.90	1.76
181 – 270	49	3.94	1.69	85	3.20	1.45
271 – 360	34	3.81	1.49	54	3.21	1.21
361 – 540	34	3.96	2.18	61	3.83	2.19
541 – 720	20	3.55	2.07	22	3.43	1.80
720 – 1080	25	3.33	1.52	4	3.13	0.64
Adults	99	3.34	0.98	–	–	–

Table XII-2. METHEMOGLOBIN REDUCTASE ACTIVITY IN RATS AS FUNCTION OF AGE

Age (days)	n	Enzyme Activity (Mean) ± S.D. as moles/min./mg.Hb
Fœtus	12	23,87 ± 2,26
3	12	15,42 ± 2,13
8	12	10,36 ± 0,65
15	11	6,56 ± 0,92
15 (males)	10	2,98 ± 0,80
25 (females)	10	4,78 ± 0,72
48 (males)	9	7,78 ± 0,35
52 (males)	10	2,65 ± 0,36
Adults (females)	6	1,91 ± 0,59

FIGURE [illegible]-1. CHANGES IN METHEMOGLOBIN REDUCTASE ACTIVITY WITH AGE - [illegible]

HEMIHYDROGEN PERIOXIDE CONCENTRATION
(% H2O2/MIN/MG HCl)
ACC (MPS)

FIGURE 4. [illegible]

for these differences are not clear. The elucidation of the mechanism of these metabolic changes will assist in the understanding of the unique fetal physiology and in formulating the precautions that should be taken when pregnant women or newborns are treated with certain drugs.

The phenomenon that MR activity during certain periods of childhood is higher than among adults has been recently reported(10). Changes in the susceptibility to oxidation with age or metabolic changes which lead to increase in production of methemoglobinemia causing factors may lead to parallel changes in MR as an adaptation defense mechanism. A similar survey done in Malaysia among different races(11) found similar MR activity in adults and newborns. The authors checked the possibility of detecting heterozygotes by total enzyme activity evaluation. They concluded that this approach is not feasible as the lower limit of normal seems to overlap with the activity found in trait carriers. We also came to these conclusions as we found a wide range of enzymatic levels in the population. The lag in the development of MR among Arab children may be associated with nutritional factors or may be of genetic origin.

Agar and Harley(12) reported recently on an MR survey among different species and the change in activity which occurs with age may have ecological meaning which is still obscure. MR activity was found to raise from 1.55 in the human fetus erythrocyte to 5.90 at the age of 6-24 months and then decrease again to the level of 1.54 which then apparently remains constant throughout life. Very similar adult human MR levels were found in several surveys done in different places of the world among different races. Different species show different ontogenetic pathway characters of MR development. In some, the enzyme activity does not change from fetus to adult; others, like the rat, show a sharp decrease with maturity. In man, MR activity doubles itself to about adult levels in the first six months of life. Beyond the interesting comparative question, these differences should be taken into account when results of toxicological experiments, in respect to the question of methemoglobinemia, are considered. The possibility that human cases of clinical methemoglobinemia in infants exposed to high nitrate concentrations in drinking water may be associated in part to MR deficiency still remains.

REFERENCES

1. Jaffe, F.A. In Biochemical Methods in Red Cell Genetics, ed. Yunis. Academic Press, New York, 1969, p 231-253.

2. Scott, E.M. In Hereditary Disorder of Erythrocytes Metabolism. E. Beutler, ed. Grune and Stratton, New York, 1968, p 102-113.

3. Ross, J.D. Deficient Activity of DPNH Dependent Methemoglobin Diaphorase of Human Erythrocytes in Cord Blood Erythrocytes. Blood 21:51-62, 1963.

4. Bartos, H.R., Des Purges, J.F., and Clark, N. Erythrocyte DPNH Dependent Diaphorase Levels in Infants. Pediatrics 37:991-993, 1966.

5. Shuval, H.I., and Gruener, N. Epidemiological and Toxicological Aspects of Nitrates and Nitrites in the Environment. Am. J. Pub. Health 62:1045-1052, 1972.

6. Gruener, N., and Cohen, S. (Unpublished data).

7. Scott, E.M. The Relation of Diaphorase of Human Erythrocytes to Inheritance of Methemoglobinemia. J. Clin. Investigation 39:1176-1179, 1960.

8. Cohen, R.J., Sachs, J.R., Wicker, D.J., and Cenard, M.E. Methemoglobinemia Provoked by Malarial Chemoprophylaxis in Viet Nam. New Eng. J. Med. 279:1127-1131, 1968.

9. Hegesh, E., Calmanovici, A., and Avron, M. New Method for Determining Ferrihemoglobin Reductase (Methemoglobin Reductase) in Erythrocytes. J. Lab. Clin.Med. 72:330-344, 1968.

10. Vetrella, M., and Barthlemai, W. Erythrocyte Enzymes in the Human Fetus. Morrotshur Kinderheilk 119:265-267, 1971.

11. Eng, L.L., Loo, M., and Fah, P.K. Diaphorase Activity and Variants in Normal Adults and Newborns. Brit. J. Haem. 23:415-425, 1972.

12. Agar, N.S., and Harley, J.D. Erythrocytic Methemoglobin Reductase of Various Mammalian Species. Experimentia 28:1248-1249, 1972.

SECTION XIII

METHEMOGLOBINEMIA INDUCED BY TRANSPLACENTAL PASSAGE OF NITRITES IN RATS

Since clinical methemoglobinemia from nitrates in water apparently appears only in infants, one possible solution proposed for areas with high nitrate water is to supply the infants with low nitrate content water from alternate sources. This measure will not exclude the risk of the exposure to nitrates in the prenatal state, i.e., the transfer of nitrates or nitrites through the placenta to the fetus, where methemoglobinemia may be induced.

The possibility of this occurring was tested on pregnant rats. Nitrites were given to pregnant rats in their drinking water or by injection and subsequently nitrite and MetHb were assayed in the fetal blood.

Suckling rats whose dams received nitrites in their drinking water showed no rise in MetHb levels. By contrast, the dams showed high MetHb levels. This demonstrates that nitrites are apparently not transferred in appreciable amounts to the suckling rats via the milk.

The transfer of nitrites to the fetus in utero and the production of MetHb was tested in the following experiment. Pregnant white albino rats were used. Each pregnant rat was weighed and anesthetized with ether. From 2.5 - 50 mg/kg of sodium nitrite was given orally or injected intraperitoneally to the pregnant rat and the kinetics of nitrites and MetHb in the dams as well as in the fetuses were measured. Blood was collected from the tail of the dam at regular intervals throughout the experiment. After opening the abdomen the fetuses were removed centally from alternate sides, at regular time intervals over a two-hour period, the umbilical cords being cauterized. The fetuses were washed in saline solution at 37°C and then decapitated. Blood was collected and the MetHb and nitrite levels were measured. The micromethods developed by our group for determining MetHb and nitrites (Sections IV and V) in blood enabled us to carry out these experiments with the small amount of blood available from each fetus.

The characteristic picture obtained is shown in Figure XIII-1. After a 30 mg/kg dose of $NaNO_2$ per os to the pregnant rat, nitrite levels rose in the fetal blood though with a lag of about 20 minutes behind the dam. This rise in nitrite in the fetus was followed by a rise in MetHb.

The possibility that the placenta was damaged during our experiments leading to increased permeability was excluded when sodium nitrite was given to normal pregnant rats after labor had started. The first fetus

showed a normal MetHb level of 1.2% MetHb while those which were born after the chemical had been given showed a level of 16.1% MetHb and 1.2 ug/ml of sodium nitrite in their blood. All births were unassisted.

Different concentrations of nitrites caused similar kinetic pictures differing only in their timing and MetHb levels. Table XIII-1 shows that the threshold of the effect was at a sodium nitrite dose of 2.5 mg/kg. The increase in effect was steep with increased dosage.

Table XIII-1 BLOOD NITRITE AND METHEMOGLOBIN LEVELS AFTER INJECTION OF DIFFERENT DOSES OF SODIUM NITRITE

$NaNO_2$ dose mg/kg	Peaks of MetHb Level as percent of total Hb		Peak of nitrite level in blood as $NaNO_2$ ug/ml	
	mother	fetus	mother	fetus
-	0.9	1.2	0.0	0.0
2.5	3.4	1.0	3.0	traces
5.0	5.0	2.7	6.9	traces
10.0	11.9	5.1	8.9	0.4
15.0	17.0	7.0	10.8	1.2
20.0	33.2	15.3	21.7	3.9
35.0	40.4	18.2	35.6	6.0
50.0	60.2	27.2	53.5	9.4

Pregnant rats exhibited a higher susceptibility to nitrites than non-pregnant rats in chronic and acute experiments.

In chronic feeding experiments pregnant rats exposed to 2000 mg/l of $NaNO_2$ in drinking water or about 200-250 mg/kg/day developed severe anemia with a mean of 10.3±1.8 g% Hb as compared with non-pregnant rats exposed to the same levels on nitrites having means of 14.2±0.9 gm% Hb. The control kept on tap water showed a mean of 14.3±0.8 g% Hb. All pregnant rats (five) died within one hour when injected with doses of 60 mg/kg while non-pregnant females survived such doses.

In general, nitrites given per os led to somewhat lower levels of MetHb as compared with comparable doses given sub-cutaneously but the kinetic picture was similar in both cases. The finding that the MetHb peak could be detected in the fetus 45-60 minutes after injecting $NaNO_2$ in the dam but not in the newborn several hours after birth indicates that the MetHb reductive mechanism in the fetus and newborn rat is highly effective. MetHb recovery rates were measured in rats after ceasing the consumption of water which contained $NaNO_2$. The time which it takes for the MetHb to be reduced to 50% of its initial level was found to be around ninety minutes, independent of the initial concentration of MetHb. NADH-dependent MetHb reductase is claimed to be the main pathway of MetHb reduction(2). The enzyme acting in the red blood cells was measured in adult rats and in fetuses. Similar determinations were made

in humans and human cord blood. Table XIII-2 shows that the rat fetuses have MetHb reductase activity some ten times higher than adult rats have or that found in human cord blood while human adult blood exhibits one and one-half times the activity of the cord blood.

Table XIII-2 METHEMOGLOBIN REDUCTASE ACTIVITY IN THE FETUS AND THE PREGNANT FEMALE - RATS AND HUMANS

	N	Methemoglobin reductase nanoles/min/mg Hb
Pregnant rats	6	1.86±0.34
Rat fetus	10	17.4 ±1.3
Human pregnant females	49	2.58±0.72
Human cord blood	69	1.58±0.04

Enzyme activity was checked in a blood sample of 10 ml which was incubated in a mixture that contains 50 nanles citrate buffer (pH4.7), EDTA 300 nanoles, $K_3Fe(CN)_6$ 56 nanoles, MetHb 208 nanoles. Total volume is 0.5 ml.

The reaction is started by adding 120 nanoles of NADH. The reduction of MetHb to methemoglobin is followed at 577 nm (Unicam SP 1800, band width of 1nm). Calculations are based on the value of 42.0 for $\Delta\epsilon M$ at 577. Molecular weight of hemoglobin is taken as 66,000. Hb was measured at 540 nm according to the cyanomethemoglobin method.

These findings point to the possibility that the human fetus might have a weaker defensive mechanism to the intra-uterine exposure to nitrites than that detected in rats.

The results underline the possible risk of intra-uterine methemoglobinemia when water or foods containing nitrates are consumed during pregnancy. It should be noted that certain foods such as spinach can contain as much as 4000 ppm of nitrates, a major portion of which can be reduced to nitrites on storage under certain conditions(5). 200 ppm of nitrites can legally be added to many "cured" meat products. It is, however, premature to extrapolate from these acute animal experiments to the situation that may exist with humans consuming MetHb inducing chemicals in water or food. A study of MetHb levels in infants' cord blood where the mothers come from areas with high and low nitrate levels in their drinking water did not reveal any raised MetHb levels among the 150 cases studied.

REFERENCES

1. Gruener, N. and Shuval, H.I., in Environmental Quality and Safety, Vol. II (Ed. by F. Coulston and F. Korte), George Thieme Verlag, Stuttgart and Academic Press, New York, p. 218, 1972.

2. Scott, E.M. in Hereditary Disorders of Erythrocyte Metabolism (edit. by Beutler, E.), 102 (Grsun and Stratton, New York, 1968).

3. Eisenberg, A., Wisenberg, E. and Shuval, H.I. The Public Health Significance of Nitrates and Nitrites in Food Products. (Ministry of Health, Jerusalem, Israel, 1970).

SECTION XIV

THE EFFECTS OF THE ADMINISTRATION OF SODIUM NITRITE IN DRINKING WATER ON PREGNANT RATS AND THEIR NEWBORN

Two experimental groups were used, each containing twelve pregnant albino "sabra" rats. Group II was given 2000 mg/l sodium nitrite and Group III 3000 mg/l in their drinking water. The control, Group I, of seven pregnant rats, received tap water without added nitrite.

The pregnant rats that received nitrites showed an increase in methemoglobinemia. Group II had a mean of 5.5% MetHb. The Group III mean was 24.0% and the mean of the Controls was 1.1%.

The pregnant rats that received sodium nitrite suffered from anemia in a direct relationship to the concentration of the compound in their drinking water. The Hb results are presented in Table XIV-1.

In view of the marked hematological effect of nitrite on the pregnant rats, we measured red cell fragility with hypotonic solutions. Erythrocytes from pregnant rats that received nitrite showed less fragility to hypotonic than those from the control rats.

Table XIV-1 DISTRIBUTION OF HEMOGLOBIN DETERMINATIONS IN PREGNANT RATS CHRONICALLY EXPOSED TO SODIUM NITRITE IN DRINKING WATER

gm% Hb	Group I Percent	Group II Percent	Group III Percent
10<	0	45.2	52.4
10.1 - 12.0	31.5	27.2	26.0
12.1 - 14.0	44.5	21.6	13.0
14.1 +	23.0	6.0	8.6

In experiments where the blood of rats was mixed with 0.0 to 0.9% sodium chloride solutions (i.e., 0 to 100% normal saline) we obtained typical sigmoidal fragility curves. In red cells, 50% hemolysis corresponded (from the curves) with 25.4% normal saline for Group III, with 30.7% normal saline for Group II and 37.8% for Group I, the controls. Thus, red blood cells from the treated groups were more resistant to hypotonicity than those from the control group.

It is possible that nitrite has some contact effect on the erythrocyte in which the "weaker" cells only are affected, the "stronger" more resistant cells remaining unaffected. Incubation of erythrocytes *in vitro* with

sodium nitrite does not have any effect on the reaction of hypotonic osmotic pressure.

MetHb is reduced in the erythrocyte to Hb with the aid of an enzyme system utilizing DPNH for this purpose:

$$\text{MetHb} + \text{DPNH} \rightarrow \text{Hb} + \text{DPN}$$

In the next experiments we test the possibility that the increase in MetHb changes the metabolic picture in the red blood cell. It is assumed that the DPNH/DPN ratio is small during the MetHb increase, due to the rapid consumption of DPNH.

The reduction of the MetHb is rapid and uptake of DPNH outbalances the rate of regeneration of DPNH in the glycolitic pathway. By way of checking the DPNH/DPN ratio we assayed the pyruvate/lactate ratio in blood from pregnant rats which, together with lactic dehydrogenase constitutes the main system in the erythrocyte using DPNH/DPN.

Lactate + DPN → Pyruvate + DPNH. Competition for DPNH between the pyruvate/lactate and the MetHb reductase systems may increase pyruvate/lactate ratio. The results are presented in Table XIV-2. The increase in the ratio was attributable to increase in pyruvate (up to 4 times more than in the control) as opposed to the small lactate increase. This points to possible increased glucose metabolism to provide extra DPNH supplies to the cells. There was a pronounced effect on mortality among newborn rats of dams receiving 2000 mg/l (Group II) and 3000 mg/l (Group III) in their water, particularly in the three-week period up till weaning.

Table XIV-2 PYRUVATE/LACTATE RATIO IN PREGNANT RATS CHRONICALLY EXPOSED TO SODIUM NITRITE IN DRINKING WATER

Group		Pyruvate/lactate ratio
I	(Controls)	0.013
II	(2000 mg/l)	0.036
III	(3000 mg/l)	0.042

The average litter in the control group contained 10 fetuses, 9.5 in Group II and 8.5 in Group III. The mortality within the first three weeks was 6% in the control as opposed to 30% in Group II and 53% in Group III. Birthweights were similar with 5.5 gm for each group. However, after the birth, newborn rats in Groups II and III lagged behind the controls in their growth rates. For example, after one week the mean weight was 16.5 gm in the control group, 12.0 gm in Group II and 9.5 gm in Group III. After 21 days (at the end of the period of giving nitrites to the dams), 51.5 gm mean weight in the control group, 29.5 gm in Group II and 16.5 gm in Group III.

Apart from the weights, a characteristic difference observed in the experimental groups was that the fur thinned and lost its luster. After separation from their dams and being put on water, there was an improvement in growth in the experimental groups. At the age of 33 days, the mean weight for the control group was 100 gm, for Group II, 67 gm and for Group III, 59 gm. At 65 days the control group attained a mean weight of 213 gm, Group I, 181 gm, and Group II, 172 gm.

During the whole period from birth to weaning, the newborn showed no abnormally high Hctlb. The mean hemoglobin of the newborn from the experimental groups was low - about 20% of the control group.

SECTION XV

INFLUENCE OF ASCORBIC ACID ON NITRATE-NITRITE INDUCED METHEMOGLOBINEMIA

INTRODUCTION

In our epidemiological survey carried out in Rehovot, Rishon-Le-Zion and Nes-Ziona, no significant difference in the mean MetHb level was found compared to the control area. One of the explanations raised was that the infants were fed some sort of antidote to methemoglobinemia. It is known that ascorbic acid is an antidote to methemoglobinemia, and as such is used in conjunction with the therapy of this disorder, especially in the congenital variety(1,2). It was found in the survey that 97% of the infants received citrus or tomato juice, and that 50% of the infants up to the age of 60 days received additional vitamin C rich supplements.

Although the possibility that ascorbic acid may be a useful prophylactic agent against methemoglobinemia has been raised before(3), experimental proof of the efficacy of this agent has been lacking. In one study(4) ascorbic acid administered in physiologic doses (50 mg/kg/day) had no effect on methemoglobin levels of healthy and distressed newborn infants. However, in this study MetHb levels were quite low to begin with (less than 1%) even in the perinatally distressed infants whose methemoglobin levels were slightly higher (0.95% as opposed to 0.755% in normals). An experimental study by Kocsha and Dlight(5) was done on acute nitrite toxicosis in ascorbic acid deficient guinea pigs. In this study subcutaneous administration of 50 mg/kg $NaNO_2$ led to higher methemoglobin levels in ascorbic acid deficient animals (Blood levels of the order of 0.55 mg%) than in controls (blood levels of the order of 0.98 mg%). Blood nitrite levels were not measured in this study.

It was decided to experimentally approach the question of ascorbic acid prophylaxis. If ascorbic acid can be shown to be a useful anti-methemoglobin prophylactic agent, ascorbic acid dietary supplementation may be indicated in areas with high nitrate loading in the environment. To begin with, it was decided to study the effects of ascorbic acid on acute nitrite induced methemoglobin, and to study the relations between blood levels of ascorbic acid, nitrite, and methemoglobin in an uncomplicated, well-studied experimental preparation.

MATERIALS AND METHODS

In all, six pairs of rats (albino Hebrew University Sabra strain) were used. They were matched for sex and weight, with weights between 400 and 450 grams. The basic experiment was done in pairs, each pair on

a different day and using new reagents each day. One rat of each pair was a control, the other the experimental animal, given the ascorbic acid. After withdrawing a sample of tail blood for ascorbic acid determination, the experimental animal was given 100 mg ascorbic acid in 1 cc of normal saline, and the control given 1 cc of normal saline, both given intraperitoneally (IP). After 1 1/2 to 3 hours, found to be the maximal absorption time in unpublished observations from this laboratory, tail blood was again withdrawn. Methemoglobin levels, ascorbic acid levels, and nitrites, were measured. Both rats were then given IP $NaNO_2$ 30 mg/kg solution. In all 6 pairs, methemoglobin and ascorbic acid levels were determined and in four out of the six pairs nitrite levels were also determined at various intervals after the injection of nitrites. Total hemoglobin levels were also taken at the beginning and the end of each experiment from each animal. MetHb, nitrites, and ascorbic acid were measured by the technique described elsewhere in this report.

Results were plotted in two ways. 1) The controls were meaned, and the experimental animals were meaned for each time interval and each variable studied. 2) Since the experiments were carried out in a paired fashion, the differences between control and experimental were meaned for each variable and each time interval. Statistical analysis was carried out on the mean differences thus determined. Statistical difference was determined by the "t" test for paired variables.

RESULTS

Figure XV-1 shows the values of the variables at each time interval after the injection of nitrite, with the controls meaned as a group, and the experimentals meaned as a group. What can be seen is that the ascorbic acid values remained relatively high in the experimental group (given the ascorbic acid), and low in the control group. Not shown are the ascorbic acid values 1-1/2 hours before the injection of nitrites, immediately prior to the injection of ascorbic acid. These were all similar to the control values shown on the figure.

It is noted that the highest value in the MetHb curve for the experimental animal occurs later and is lower than the highest value for the control animal. However, after one hour the two curves become very similar to each other.

It is further noted that the nitrite curves seem to be parallel in a way to the methemoglobin curves, except that their peaks are earlier. The control nitrite peak was higher and earlier than the experimental, just as in the case of the methemoglobin peaks. After 20-25 minutes the two curves become quite similar to each other.

The data are shown analyzed in a paired fashion in Figures XV-2 and XV-3. Figure XV-2 shows the mean differences (control minus experimental)

FIG. 1.

MEAN ASCORBIC ACID, METHEMOGLOBIN AND NITRITE LEVELS IN 6 RATS ADMINISTERED 20 mg/kg $NaNO_2$ AT TIME ZERO

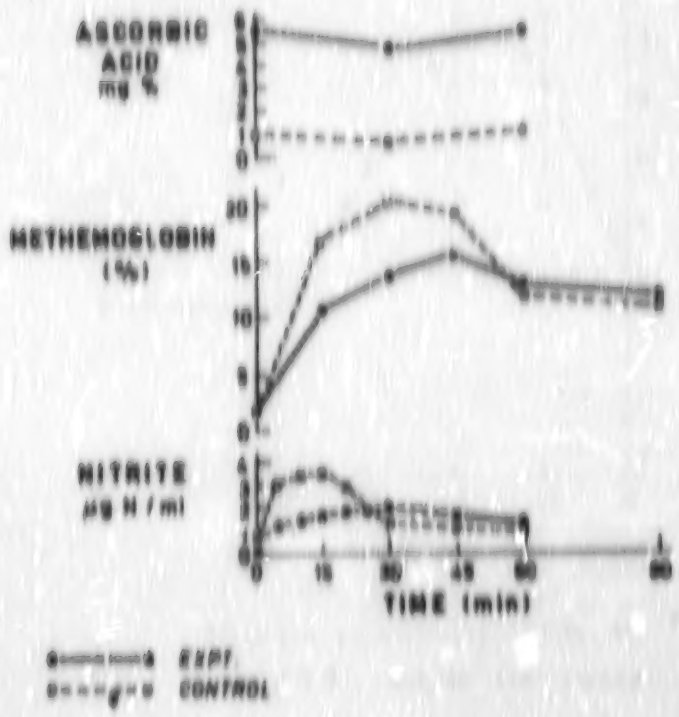
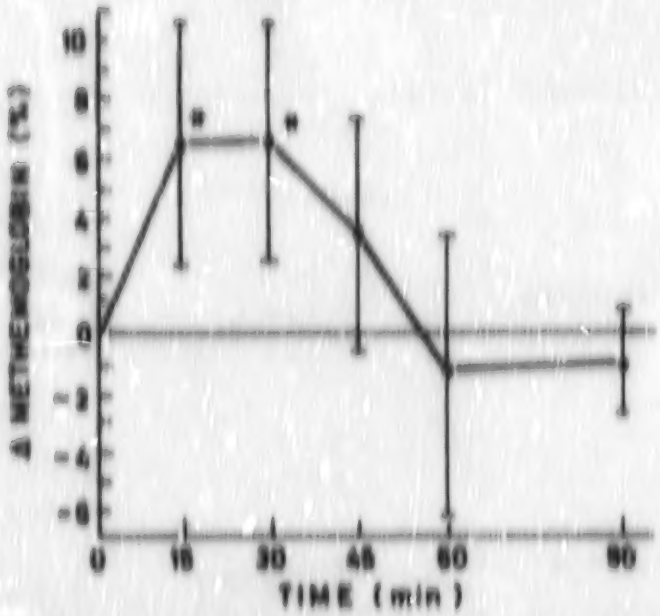


Figure XV-1. MEAN ASCORBIC ACID, METHEMOGLOBIN, AND NITRITE LEVELS IN RATS ADMINISTERED 20 mg/kg $NaNO_2$ AT TIME ZERO.

FIG. 3

MEAN DIFFERENCE BETWEEN EXPERIMENTAL AND CONTROL ANIMALS (CONTROL MINUS EXPERIMENTAL) IN METHEMOGLOBIN LEVELS AT EACH TIME POINT STUDIES



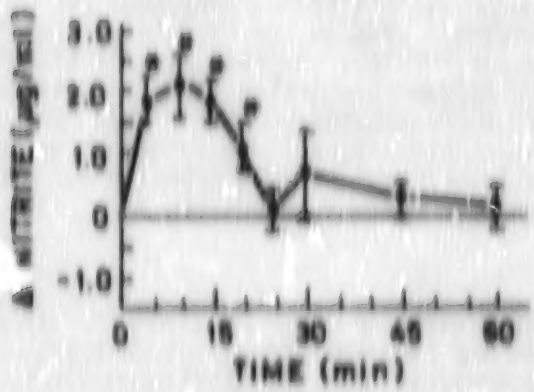
* statistical significance $p \leq 0.05$

shown are means ± 1 SD

Figure XV-3. MEAN DIFFERENCE BETWEEN EXPERIMENTAL AND CONTROL ANIMALS (CONTROL MINUS EXPERIMENTAL) IN METHEMOGLOBIN LEVELS AT EACH TIME POINT STUDIES.

FIG. 3

MEAN DIFFERENCE BETWEEN EXPERIMENTAL AND CONTROL ANIMALS (CONTROL MINUS EXPERIMENTAL) IN NITRITE LEVELS AT EACH TIME POINT STUDIES



* statistical significance $p \leq 0.05$
shown are means ±1 S.D

Figure XV-3. MEAN DIFFERENCE BETWEEN EXPERIMENTAL AND CONTROL ANIMALS (CONTROL MINUS EXPERIMENTAL) IN NITRITE LEVELS AT EACH TIME POINT STUDIES.

between the methemoglobin values at each time interval after the injection of nitrites. These are maximum at 15 and 30 minutes. After 60 minutes the mean difference becomes small and insignificant. At 45 minutes the mean difference is sizable, but not significant, probably due to the smallness of the sample.

Figure XV-5 shows a plot of the mean differences in nitrite values (control minus experimental) for each time interval following nitrite injection. This curve resembles the methemoglobin curve in its biphasic nature. There is a period of large difference followed by one of small, insignificant difference. The highest difference in nitrite values occurs earlier than the highest difference in methemoglobin values.

Thus, if one uses the mean difference between the control and the experimental animals as an index of the protective effect of ascorbic acid, the following may be said: 1) The main protective effect of ascorbic acid appears in the first, and highest, portion of the methemoglobin formation curve, up to 60 minutes post nitrite injection. After that protection seems to be lacking. The protective effect seen here was of the order of magnitude of 6-7% methemoglobin less in the experiment than in the control out of a total of 20% in the control. 2) This pattern seemed to parallel the nitrite pattern, with maximum protective effect on the first and highest portion of the curve, up to 30-35 minutes. After that the values between control and experiment were very similar. It should be noted that the difference in ascorbic acid was high between experimental and control, at least up to 60 minutes post nitrite injection.

DISCUSSION

The mechanism of any protection given by ascorbic acid against nitrite induced methemoglobinemia may be either direct or indirect. Direct reduction of methemoglobin by ascorbic acid certainly does occur, but is a slower process than the enzymatic reduction(6). Indirect mechanisms include increase in reductase activity, blocking of nitrite binding sites, and lowering of blood nitrite levels. From these data presented here the question cannot be decided. However, there is suggestive evidence that the ascorbic acid may act by lowering the peak nitrite levels. There is a striking parallel between the nitrite and the methemoglobin curves. That nitrites rise and fall sooner than the corresponding methemoglobin curves is known in this laboratory from many previous experiments, as well as the fact that the methemoglobin levels are directly proportional to the corresponding nitrite levels. From the data here it seems quite reasonable to postulate that the primary effect of ascorbic acid is to lower the blood nitrite levels in the earliest, maximal portion of the curve, the methemoglobin levels being consequent to this action. In the latter portion of the curve, when the nitrite levels are very similar for the control and the experimental groups, and the ascorbic acid levels are still high in the experimental animals, there is a corresponding similarity between control and experimental methemoglobin levels.

As to why the nitrite peak is lower with ascorbic acid, one is left with mere speculation. Three possibilities come to mind: 1) Nitrite and ascorbic acid combine directly in some sort of complex, effectively removing the nitrite from either the absorptive peritoneal surface or the blood stream. 2) Ascorbic acid prevents nitrite absorption in some other manner, possibly by peritoneal irritation. 3) Ascorbic acid, in some fashion, hastens nitrite detoxification and withdrawal through enzymatic or non-enzymatic means.

Possible public health implications depend upon several considerations. First of all, simply looking at the shape of the methemoglobin curves, without regard to mechanisms, several facts are apparent. 1) There is partial protection against methemoglobinemia in the earliest portion of the curve. There is about 1/3 less methemoglobin measured as percent total hemoglobin in the experimentals as compared with the controls. 2) This protection is not complete even with ascorbic acid blood levels quite high of the order of 5-6 mg%. 3) After one hour the protective effect seems to be lost, and if anything, the methemoglobin values in the experimental are slightly, though not significantly, higher. The question naturally arises if in chronic exposure to high nitrites or nitrates which convert to nitrites the methemoglobin levels behave as if they were on the first or second portion of the curves. Preliminary experiments in this laboratory indicate that high vitamin C intake does lower the methemoglobin blood level in mice exposed to high nitrite doses. However, a great deal of work remains to be done in this area. If ascorbic acid, with chronic administration of high nitrites, or nitrates, causes the methemoglobin levels to behave as if they were on the second portion of the curve, it is apparent that administration of high doses of ascorbic acid will not completely prevent methemoglobinemia, but may nevertheless provide a certain degree of easily obtainable protection.

REFERENCES

1. Faivre, M., Armand, J., and Faivre, J. Les methemoglobinemies toxiques. Masson C., ed. Paris, 1970. p. 102.

2. Fitch, J., Walker, G., and Baxter, B. Methemoglobinemia - a cause of cyanosis in infants and children. Arch. Ped. 66:143-156, 1949.

3. Gruener, N., and Shuval, H.I. Health aspects of nitrites in drinking water. In: Developments in Water Quality Research. Proc. Jerusalem International Conf. on Water Quality and Pollution Research. Jerusalem, 1969.

4. Pihlajo, T., Valmaki, I., and Yrjana, T. Effects of peroral ascorbic acid on blood methemoglobin of newborn infants. Biol. Neonat. 13:63-67, 1968.

5. Kociba, R.J., and Sleight, S.D. Nitrite toxicosis in ascorbic acid deficient guinea pig. Toxic and Appl. Pharm. 16:424-429, 1970.

6. Gibson, Q.H. The reduction of methemoglobin by ascorbic acid. Biochem. J. 37:615, 1943.

SECTION XVI

CHRONIC EXPOSURE OF RATS TO SODIUM NITRITE IN DRINKING WATER

Previous chronic studies by others have not shown significant MetHb levels or other pathological changes in rats consuming water containing nitrites in the range of 500-2,000 mg/l(1,3).

We have shown that rats being nocturnal animals consume 80% of their water at night, and because of the rather rapid rate of the MetHb reduction, by 10:00 A.M. they may no longer show significant MetHb levels even when consuming water high in nitrites. Our measurements showed peaks in water consumption and MetHb level in the middle of the night. In designing our chronic studies, we adjusted the "day" and "night" hours in the animals' rooms so that blood examinations would be taken at about the rats' "midnight," in order to detect the expected maximum daily concentration of MetHb.

Based on the information gained from our first chronic toxicity studies, in which 40 male rats divided into 5 groups, were exposed to nitrite, for two years, ranging from 100-3,000 mg/l, we have designed a larger comprehensive experiment to follow chronic toxicity of rats exposed to sodium nitrite as well as sodium nitrate. The total number of rats included in this study was 312. Four groups (II, III, IV, VI) exposed to 200; 1,000; 2,000; 3,000 mg/l of $NaNO_2$ and one group (VI) exposed to 3,000 mg/l of $NaNO_3$ as well as a control group (I) of the same size were included in this study. In each group there are 52 male albino Hebrew University Sabra rats. The following parameters were checked at regular intervals:

1. Water consumption; 2. Body weight; 3. Methemoglobin(MetHb); 4. Hemoglobin (Hb); 5. Hematocrite; 6. MB; 7. Serum lactic dehydrogenase; 8. Serum glutamic acid; 9. Blood glucose; 10. Nitrite in blood and 11. Diphosphoglyceric acid in red blood cells.

Histological examinations were carried out on heart and lung taken from rats of the six groups sacrificed at 3-month intervals.

A special animal room for chronic studies was prepared with controlled light, temperature and ventilation. After weaning, animals were held in the new conditions for one month for a period of adaptation prior to being exposed to the various experimental conditions.

ANALYTICAL METHODS

1. Methemoglobin determination:

   This was done according to our method(1).

2. Hemoglobin was determined by the cyano-methemoglobin method(4).
3. Hematocrite was determined by the micromethod using a "Hawksley" centrifuge.
4. Methemoglobin reductase was assayed as described in this report.
5. Glutamic acid levels in serum were determined according to Bernt & Bergmeyer(5).
6. Blood glucose was determined with the toluidine method(6).
7. Nitrites in blood were tested according to our method(7).
8. 2,3 Diphosphoglyceric acid (DPG) was assayed enzymatically as follows:

PRINCIPLE:

a) 2,3 Diphosphoglycerate $\xrightleftharpoons[\text{2-phosphoglycolic acid}]{\text{Phosphoglyceromutase}}$ 3 phosphoglycerate

b) ATP + 3 phosphoglycerate $\xrightleftharpoons{\text{Phosphoglycerate Kinase}}$ 1,3-diphosphoglycerate + ADP + H^+

c) 1,3 diphosphoglycerate + NADH $\xrightleftharpoons{\text{Glyceraldehyde -P dehydrogenase}}$ Glyceraldehyde -3-P + NAD + PO_4

The phosphatase activity of muscle phosphoglycerate-mutase is enhanced by phosphoglycolic acid. Phosphoglycerate is formed from DPG and is phosphorylated to 1,3 diphosphoglycerate (reaction b) which is reduced to the aldehyde. Reduction of NADH absorbance is followed at 340 mμ. Standards of DPG were tested in each run.

<u>Reagents</u>

1. Buffer solution; contains 1.567 gm Imidazol, 1.017 gm $MgCl_2$, 0.651 gm Hydrazine sulfate, and 0.472 mg 2-Phosphoglycolate per liter distilled H_2O. Solution is adjusted to pH of 7.4, May be refrigerated and kept stored.

4. Curvette mixture. Must be made daily. Does not store. Contains for each 10 ml buffer solution, 50 mg Adenosine triphosphate (ATP) 35 mg reduced glutathione (GSH), 5 mg Diphosphopyridine nucleotide reduced form (DPNH), 0.1 ml Glyceraldehyde-3-phosphate dehydrogenase (Ga-3-PD) and 0.05 ml 3 Phosphoglycerokinase (PGK).

5. Phosphoglycerate - Mutase (PGM).

Procedure

1. Add 0.1 ml whole blood to 0.3 ml distilled water, mix well, and take a sample for Hb determination.

2. Put the tube in boiled water for five minutes and centrifuge for 2 minutes at 15,000 RPM.

3. To spectrophotometer curvette, add 0.1 ml supernatant and 0.9 ml curvette mixture. Substitute 0.1 ml distilled water for blank.

4. Read at 340 nm and do not start reaction until each curvette has reached stability. Note this value as time 0.

5. To each curvette, add 0.005 ml PGM. Shake well.

6. Read until DPNH falls at the same rate as with water blank.

RESULTS

The average 24 hours liquid intake is presented in Table XVI-1. There was essentially no change in the liquid intake expressed as mg/kg/day throughout the experiment.

Table XVI-2 shows the change in body weight during the experiment. The daily range of nitrites or nitrates which the experimental groups received can be seen in Table XVI-3.

It can be seen from Table XVI-2 that only the rats that got the highest nitrite quantities suffered from a slight retardation in growth and development. They also showed some rejection of the drinking solution.

It is worth noting here that the highest nitrite groups received a daily amount which is about twice the single dose LD_{50} of sodium nitrite for adult rats (around 150 mg/kg). This exposure nevertheless did not cause far-reaching physiological deviations. It appears that some form of nitrite detoxification and compensatory mechanisms are strong enough to cope with such levels of exposure when given in many small doses throughout the day. Our other studies indicate that suckling rats are more sensitive.

Table XIV-1. Liquid intake of male albino rats [illegible]

[illegible] lbs.

Group	Time of Exposure		3 weeks	6 weeks	12 weeks	4 months	18 months	24 months
I	Control	n	(19)	(19)	(19)	(19)	(9)	(9)
		M	38.7	55.5	46.6	46.5	38.1	37.1
		S.D.	3.6	2.8	5.6	5.5	4.7	4.6
II	200 mg/l $KMnO_4$	n	(19)	(19)	(19)	(19)	(9)	(9)
		M	38.7	45.9	42.4	42.9	42.2	37.5
		S.D.	3.6	4.7	5.2	4.7	5.6	4.8
III	1000 mg/l $KMnO_4$	n	(19)	(19)	(19)	(19)	(9)	(9)
		M	38.9	44.7	40.9	42.7	37.9	34.4
		S.D.	5.4	3.7	5.6	4.9	5.7	5.9
IV	2000 mg/l $KMnO_4$	n	(19)	(19)	(19)	(19)	(9)	(9)
		M	35.1	40.3	35.2	38.2	34.9	34.9
		S.D.	3.7	5.5	5.1	5.2	5.8	5.6
V	3000 mg/l $KMnO_4$	n	(19)	(19)	(19)	(19)	(9)	(9)
		M	32.7	35.2	32.9	34.1	32.9	34.9
		S.D.	4.9	5.4	4.6	4.6	5.2	5.6
VI	5000 mg/l $KMnO_4$	n	-	(19)	(19)	(19)	(9)	(9)
		M		47.7	45.7	44.9	43.6	42.9
		S.D.		5.4	5.5	5.5	5.9	5.9

Table XVI-3 shows the change in body weight during the experiment. The daily range of nitrites or nitrates which the experimental groups received can be seen in Table XVI-5.

It can be seen from Table XVI-3 that only the rats that got the highest nitrite quantities suffered from a slight retardation in growth and development. They also showed some rejection of the drinking solution.

It is worth noting here that the highest nitrite groups received a daily amount which is about twice the single dose LD_{50} of sodium nitrite for adult rats (around 180 mg/kg). This exposure nevertheless did not cause far-reaching physiological deviations. It appears that some form of nitrite detoxification and compensatory mechanisms are strong enough to cope with such levels of exposure when given in many small doses throughout the day. Our other studies indicate that suckling rats are more sensitive.

Table XVI-4 represents the mean MetHb levels. Groups III, IV and V were significantly raised throughout the study. Group III showed what might be considered subclinical levels, while Groups IV and V showed MetHb levels which would be considered in humans as clinically significant.

Table XVI-5 represents steady-state levels of nitrites in blood. There is a low level NO_2^- in the control blood which does not increase in the lowest exposure group (200 mg/l of $NaNO_2$) but rises proportionally as the levels of exposure increase to the highest exposure group. It is worth mentioning that a raised level of nitrites in blood was found in Group VI which received only $NaNO_3$. This group did not however show raised MetHb.

Table XVI-6 represents the peaks of MetHb as caused by different single dosages of sodium nitrite. There is a clear dose-response rela- The kinetics of MetHb change after a single administration of $NaNO_2$ resulting in an increase phase of 30 min. to 90 min. which culminates in a sharp peak and declines in a long recovery phase with complete recovery after about five hours.

The kinetics of the recovery follow a first order reaction with a $t_{1/2}$ of about 80 min. A characteristic recovery picture can be seen in Figure XVI-1. Methemoglobin reductase which is the main mechanism of MetHb reduction was measured throughout the experiment (Table XVI-7). The level of the enzyme activity shows fluctuations with age. When levels are compared among groups at each time of the determination there is a definite decrease in the enzyme activity in the groups exposed to nitrites after a lag period of one month of exposure and in recovery to to control levels toward the end of the experiment (16 months).

Table XXI-2. CHANGES IN BODY WEIGHT IN RATS DRINKING SPIKED NITRITE AND NITRATE

(grams)

Group	Time of Exposure	Zero time (8 weeks of age)	10 weeks	20 weeks	10 months	14 months	16 months
I	n	(52)	(52)	(52)	(36)	(35)	(28)
	$\bar{X}$	243.9	308.9	437.2	526.6	542.1	602.8
	S.D.	13.6	26.7	34.4	48.5	67.7	65.4
II	n	(52)	(52)	(52)	(36)	(36)	(28)
	$\bar{X}$	241.8	325.6	442.7	502.9	535.3	590.2
	S.D.	8.8	25.6	36.9	43.2	52.3	±54.6
III	n	(52)	(52)	(52)	(36)	(36)	(28)
	$\bar{X}$	228.2	321.8	467.9	536.3	538.8	602.5
	S.D.	±2.4	±26.2	±32.8	±45.9	±54.8	±55.6
IV	n	(52)	(52)	(52)	(36)	(36)	(28)
	$\bar{X}$	226.8	313.4	434.8	529.5	557.9	606.2
	S.D.	±2.9	±33.3	±44.9	±56.8	±69.1	±57.1
V	n	(36)	(36)	(48)	(31)	(29)	(25)
	$\bar{X}$	228.4	304.9	407.5	485.3	508.6	544.8
	S.D.	±1.4	±38.5	±48.8	±44.2	±40.9	±45.1
VI	n	-	-	(52)	(48)	(38)	(32)
	$\bar{X}$			458.8	538.6	575.9	624.4
	S.D.			±55.7	±51.76	±96.6	±58.8

[illegible]

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

[illegible]

Table XVI-5. NITRITE LEVEL IN RAT BLOOD EXPOSED TO SODIUM NITRITE AND NITRATE ug/ml

Group	n	Mean	S.D.
I	8	0.15	0.38
II	30	0.10	0.08
III	28	0.44	0.42
IV	26	0.76	0.67
V	24	1.14	0.73
VI	19	0.44	0.15

Table XVI-6. METHEMOGLOBIN IN RATS AFTER A SINGLE DOSE OF $NaNO_2$-I.P.

Dose (mg/kg)	Time of max. (min)	Max. Metlb (%)
10	30	7.1
20	40	20.3
40	75	36.7
60	90	51.4
80	90	67.5 (died)

Table [illegible]. [illegible] ACTIVITY IN RATS EXPOSED

TO $NaNO_2$ AND $NaNO_3$

([illegible])

Group		Time of Exposure (months)				
		0.5	1.0	2.0	4.0	16.0
I	n	(8)	(15)	(50)	(48)	(24)
	$\bar{X}$	2.5	2.1	1.7	3.8	4.8
	S.D.	0.2	0.4	0.7	0.4	1.8
II	n	(9)	(36)	(51)	(47)	(29)
	$\bar{X}$	2.9	2.2	1.7	2.7	5.5
	S.D.	0.5	0.4	0.8	0.8	4.2
III	n	(8)	(15)	(48)	(48)	(25)
	$\bar{X}$	2.5	2.4	1.4	1.9	5.8
	S.D.	0.3	0.4	0.6	0.9	2.1
IV	n	(8)	(15)	(46)	(46)	(25)
	$\bar{X}$	2.3	2.0	1.3	2.3	4.6
	S.D.	0.4	0.5	0.7	0.8	1.5
V	n	(9)	(36)	(48)	(41)	(24)
	$\bar{X}$	2.2	7.5	0.6	2.1	5.8
	S.D.	0.6	0.6	0.5	0.6	2.6
VI	n	-	-	(25)	(7)	(31)
	$\bar{X}$			1.9	2.6	4.5
	S.D.			0.8	0.5	1.4

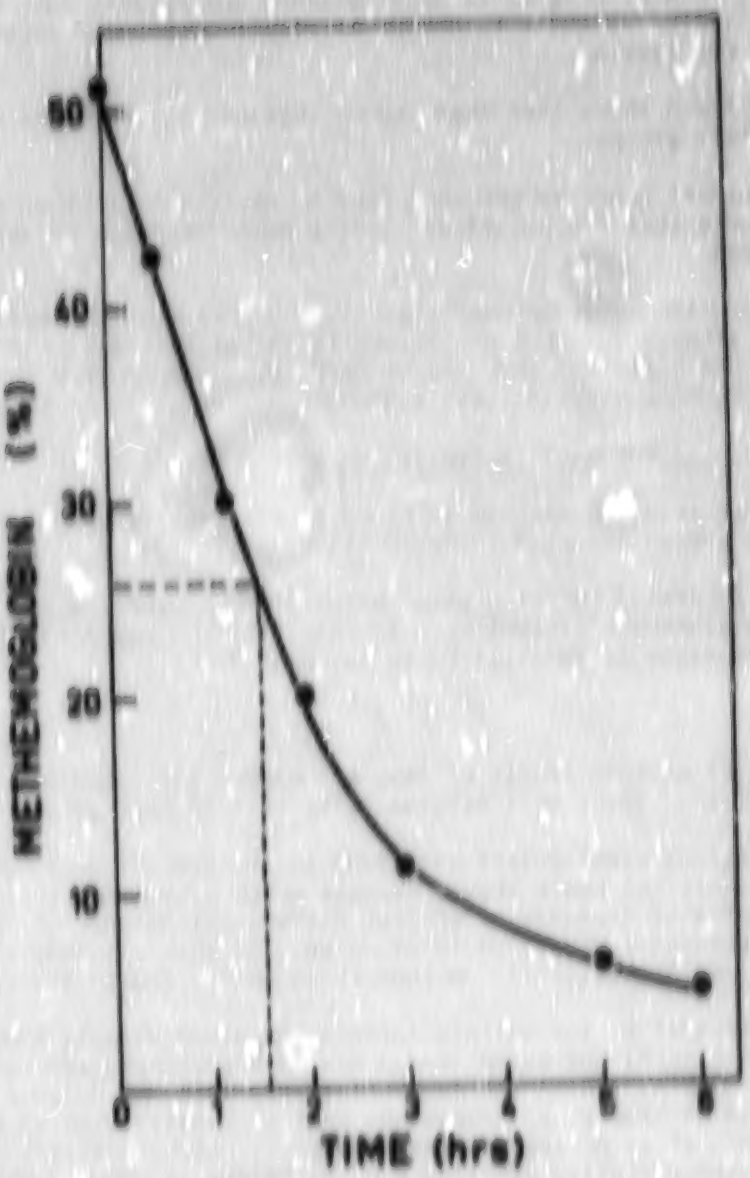


FIGURE XVI-1. METHEMOGLOBIN REDUCTION RATE IN RAT.

Diphosphoglyceric acid (DPG) levels

Concentrations of DPG in red blood cells are increased in conditions of hypoxia, anemia, congenital heart disease and chronic lung diseases(9). It was considered relevant to study the effect of induced methemoglobinemia on DPG levels.

Table XVI-8 shows that there is an increase in DPG level in the higher nitrite groups.

Preliminary acute experiments done to study the kinetics of DPG changes showed that 2-3 hrs after nitrite administration no change in DPG could be seen.

Further experiments showed that after 24 hrs changes could be detected and after 3 days DPG stabilized at the higher level and remained so throughout the exposure time. After $NaNO_2$ was removed from the drinking water the DPG concentration levels dropped but at a slower rate.

Glutamic acid in chronically treated rats

Glutamic acid was checked in blood of the experimental rats and the results are represented in Table XVI-9.

Blood incubated in vitro with sodium nitrite caused a significant decrease in glutamate metabolism. If this fact has any relevance to the chronic phenomenon, it is still to be elucidated.

Pathology

Deaths of unknown origin of rats throughout the experiments are shown in Table XVI-10. There were originally 52 rats in each group.

Histological examinations were done in the pretest on different tissues but only the heart showed changes which called for further studies. Up to 12 months of exposure no special differences between the control and the experimental groups could be shown. At this age some change was noted which was significantly obvious at 18 months (Table XVI-11).

While in most of the control animals the blood vessels showed some degree of thickening and often even a marked hypertrophy and narrowing, in the experimental groups the coronaries were thin and dilated (Figures XVI-2 and XVI-3), their appearance not what is usually seen in animals of advanced age. It is noteworthy that Group VI, which received sodium nitrate, showed a similar prevalence of pathology as Group V, which was exposed to sodium nitrite.

Table XVI-8. DIPHOSPHOGLYCERATE LEVEL IN ERYTHROCYTES OF RATS EXPOSED TO $NaNO_2$ AND $NaNO_3$

µmoles/gHb

Group	n	mean	S.D.
I	11	12.7	1.6
II	7	14.6	3.6
III	8	14.7	1.8
IV	12	16.8	4.5
V	7	17.9	4.5
VI	7	12.6	2.4

Table XVI-9. GLUTAMATE LEVELS IN BLOOD OF RATS EXPOSED CHRONICALLY TO SODIUM NITRITE AND NITRATE

(m µmoles/ml)

	I		II		III		IV		V		VI	
n	21		25		21		22		23		31	
mean	242.2	29.8	307.7	46.1	310.3	75.6	326.0	50.3	576.8	60.6	337.1	66.5

Table XVI-10. DEATHS OF UNKNOWN ORIGIN AMONG RATS TREATED WITH SODIUM NITRITE AND NITRATE

Group	I	II	III	IV	V	VI
n	2	-	2	1	0	-

Table 20-11. THE EFFECT OF [illegible] ACTIVITY ON [illegible] SCORES OF RATS EXPOSED FOR 24 HOURS

Group	I		II		III		IV		V		VI	
Dose of [illegible]	Control		[illegible] mg/l		[illegible] mg/l		[illegible] mg/l		[illegible] mg/l		[illegible] mg/l	
Total number	n	%	n	%	n	%	n	%	n	%	n	%
Normal	34	67	12	52	3	15	4	21	3	18	3	15
Tics	4	19	11	48	15	50	13	45	12	71	10	85
Tics/A	15	71	6	26	4	25	8	42	7	41	5	20
Total*	21		23		26		19		17		20	

*Total number of animals. The same beast may show more than one stereotypy in the corresponding [illegible]

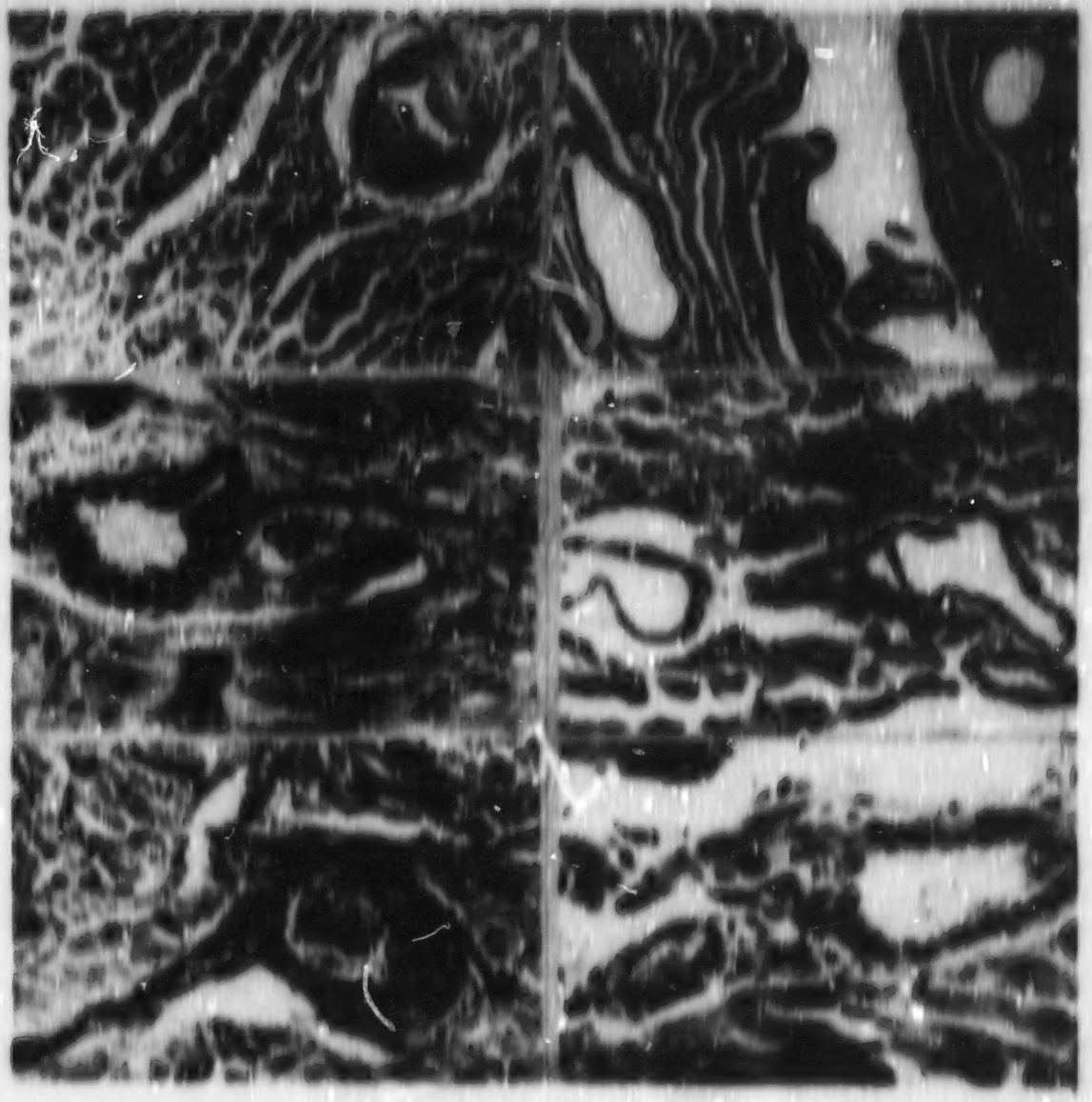


Figure XVI-4. HEART, CONTROL, 18 MONTHS OLD RAT.

Figure XVI-5. HEART, RAT, AFTER 18 MONTHS OF DRINKING WATER CONTAINING 1000 ppm $NaNO_2$

Animals were checked routinely for Hb, hematocrit, blood glucose, and serum GOT. No differences were detected between the various experimental groups and the controls.

DISCUSSION

In spite of the fact that rather high dosages of sodium nitrite were given to the rats in Groups III, IV and V, only in the last group could clinical symptoms be detected. Death rate in this Group (V) was higher than in the control but not among the other groups. The daily intake of $NaNO_2$ of Groups IV and V were higher than the single dose LD_{50} of $NaNO_2$ but the animal could handle these quantities without extreme deviations from their normal physiology. This fact exhibits the efficacy of the elimination and recovery mechanism toward nitrite. One such compensatory mechanism which was described in this work is the increase in DPG in groups showing nitrite induced methemoglobinemia. This phenomenon of increased levels of DPG have been described in different types of hypoxia(8). We can hypothesize that this phenomenon is a result of methemoglobinemia and the direct effect of nitrites themselves. Such an hypothesis is strengthened by a recent note(9) that increased levels of DPG were detected in a brother and sister who suffered from congenital methemoglobinemia. The triggering mechanism for this compensatory reaction is unknown as the regulatory mechanism in other hypoxic states. It was found that oxyhemoglobin has half of the affinity to DPG as deoxyhemoglobin(10). It was suggested that this binding reduces soluble DPG levels and causes an increase in glycolysis and thus higher levels of DPG(11). Such a mechanism can apply also to MetHb. Relative affinity of DPG to MetHb and to other Hb species should be compared. Binding of DPG to MetHb has already been shown(12).

Another possibility of regulating DPG by MetHb is by affecting the glycolytic pathway rate. Higher consumption of NADH for reduction of MetHb may stimulate glyceraldehyde-phosphate dehydrogenase to produce more 1,3 diphosphoglycerate and hence more DPG.

As can be seen from Table XVI-8 there is a correlation between glutamic acid levels and sodium nitrite intake (and hence, levels of nitrite in serum, Table XVI-5). Preliminary experiments carried out in vitro showed that the presence of nitrite causes a reduction in the metabolism of glutamic acid in red blood cells. Whether this observation had relevance to the chronic changes and what are the enzymes affected is still to be elucidated.

Glutamic acid is known to be a powerful excitator of neurons. There is a possibility that nitrite will cause accumulation in brain glutamic acid. If such a possibility will be verified it well may be an explanation to the central effects of nitrites described in this report.

The pathological indications in coronary blood vessels of rats exposed to sodium nitrite and nitrate has proved to be one of the few clear-cut

effects of chronic exposure to these chemicals. It is particularly noteworthy that the effect was just as prevalent in the rats exposed to sodium nitrate as sodium nitrite although the former showed no signs of raised MetHb throughout the experiment. That these pathological findings were clearly detected in the group exposed to the lowest level of $NaNO_2$ is of particular concern since that group received only from 15-30 $\mu g/kg$/day. Such an exposure level is not far from the exposure that could occur in humans under certain, not too extreme, conditions. The significance of this type of coronary pathology in human health is not clear. To what extent these findings may be associated with the reported higher prevalence of heart disease in areas supplied by drinking water with high nitrate concentrations(14) must be fully investigated.

REFERENCES

1. Druckrey, H., Steinhoff, D., Beuthner, H., Schneider, H., Kissner, F., Screening of Nitrite for Chronic Toxicity in Rats. Arzneimittel Forsch. 15, 520-525, 1965.

2. Mosil, J. The Effect of a Chronic Sodium Nitrite Intoxication on Rats. Acta Biol. Med. German, 10, 388-394, 1966.

3. Hegesh, E., Gruener, N., Cohen, S., Buchkovsky, S., R. and Shuval, H.I., A Sensitive Micromethod for the Determination of Methemoglobin in Blood. Clin. Chim. Acta, 30, 679-682, 1970.

4. Drabkin, D.L. and Austin, J.H. Spectrophotometric Studies; Preparation from Washed Blood Cells; Nitric Oxide Hemoglobin and Sulfhemoglobin, J. Biol. Chem., 112, 51-65, 1935.

5. Bernt, E. and Bergmeyer, H.U. Determination of L-glutamate in Method of Enzymatic Analysis (H.U. Bergmeyer, ed.) pp. 384-388, Verlag Chemie and Academic Press, Weinheim and New York.

6. Ayvärinen, A. and Nikkila, E.A. Specific Determination of Blood Glucose with O-toluidine, Clin. Chim. Acta, 7, 140, 1962.

7. Ghechter, H., Gruener, N., and Shuval, H.I. A Micromethod for Determination of Nitrite in Blood. Anal. Chim. Acta, 60, 95-99, 1972.

8. Brewer, G.J. and Eaton, G.W. Erythrocytic Metabolism: Interaction with Oxygen Transport. Science, 171, 1305-1311, 1971.

9. Kühler et al. Congenital NADH-Dependent Methemoglobin Reductase Deficiency in Combination with Glycerol Phosphate Dehydrogenase Activity in Red Blood Cells. Blood, 40, 459, 1972.

10. Garby, L., Gerber, G.H., and de Verchier, C.H., Binding of 2-3-Diphosphoglycerate and Adenosine Triphosphate to Human Hemoglobin A. Eur. J. Biochem., 10, 110, 1969.

11. Lenfant et al. Effect of Altitude on O_2 Binding by Hemoglobin and on Organic Phosphate Levels. J. Clin. Invest. 47, 2652, 1968.

12. MacQuarrie, and Gibson, Q.H. The Effect of Organic Phosphates on Methemoglobin. Fed. Proc. 30, 572, 1971.

13. Krnajevic, K. and Phillis, J.W. Iontophoretic Studies of Neurons in the Mammalian Cerebral Cortex. J. Physiol. (London) 165, 274.

14. Morton, M.E. Am. J. Pub. Health, 61:1571, 1971.

SECTION XVII

THE REDUCTION OF METHEMOGLOBIN IN THE HUMAN ERYTHROCYTE

The main reduction pathway of MetHb in the erythrocyte is regarded as NADH dependent(1). Several enzymatic preparations have been isolated using mainly NADH for MetHb reduction(2-4). This report describes the pattern of enzymatic activity which shows rather unusual features.

The source of MetHb and the enzymatic preparation was human outdated blood. The washed erythrocytes were hemolysed in water and the membrane fraction was centrifuged at high speed. The supernatant fluid was dialysed overnight against phosphate buffer (10mM; pH 7.0). The dialysed solution was placed on a DEAE cellulose column (0.9 x 15 cm) on which the enzyme was absorbed and the hemoglobin (Hb) eluted with phosphate buffer (10 mM, pH 7.0). The Hb solution was checked each time and found free of MetHb reductase activity. The enzyme was eluted from the column with a gradient of KCl solution (40-200mM).

MetHb reductase assay I. The reduction of MetHb to oxyHb was followed at 577nm at 25°C. A Unicam sp 1800 with a bandwidth of 1nm was used. The incubation mixture contained 50 μmoles of tris buffer (pH 7.4) or 40 μmoles of citrate buffer (pH 4.7); 50 m μmoles of EDTA; 130 m μmoles of NADH; 50-250 μg of enzyme preparation. Different concentrations of MetHb together with potassium ferrocyanide were present in the assay in the molecular ratio of 4:1 ($Fe(CN)_6^{4-}$: MetHb). The mol wt reading of MetHb was 66,000. Total volume was 0.6 ml.

MetHb reductase assay II. The reaction mixture was as described in Assay I. Samples were withdrawn up to 15 min. in 2 1/2 min. intervals from the incubation mixture. The percentage of MetHb was determined by our method(5). The percentage values were converted into chemical concentrations on the base of total Hb value determined by the cyanomethemoglobin method(6). Enzymatic activity was calculated according to the method of Lee and Wilson(7). Protein was determined according to the method of Lowry et al.(8).

Sigmoid curves are obtained by plotting enzyme activity vs. MetHb concentration at low and high pH (Figures XVII-1 and XVII-2). The Hill coefficient -n(9) was in the range of 2 to 2.2 in different experiments at both high and low pH. The $S_{0.5}$ was about 50 μM at the acidic pH, and about 500 μM at the neutral one.

Hebesh and Avron reported low activity at pH 7.4 compared with "optimal" pH 5.0(3). This was true only at rather low concentrations

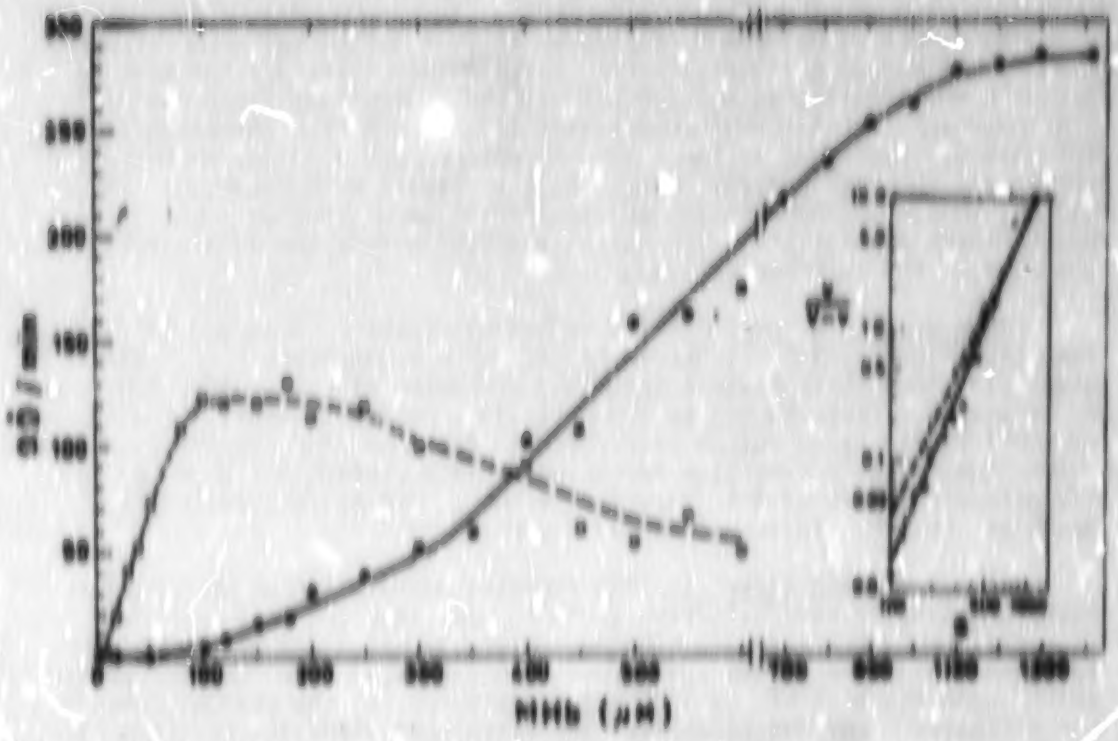


Figure XVII-1 EFFECT OF MMb CONCENTRATION ON METHEMOGLOBIN REDUCTASE ACTIVITY. Hill plot of the results is shown in the insert.
(o-o-o in the presence of buffer citrate pH 4.7)
(o-o-o in the presence of buffer tris pH 7.4)

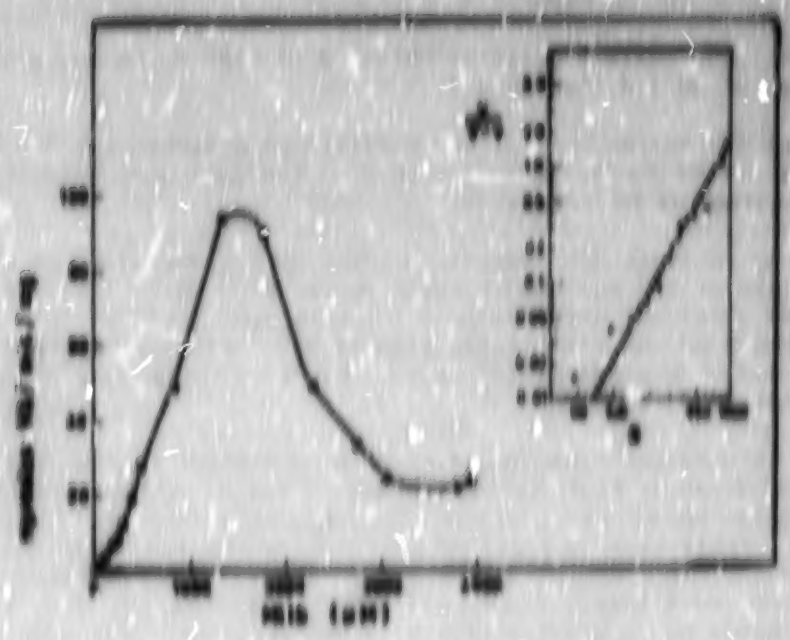


Figure XVII-2. Effect of the MMb concentration on methemoglobin reductase activity. Fill plot of the results is shown in the insert. The reaction mixtures were of the composition described in Assay II. Buffer tris (pH 7.4) was used.

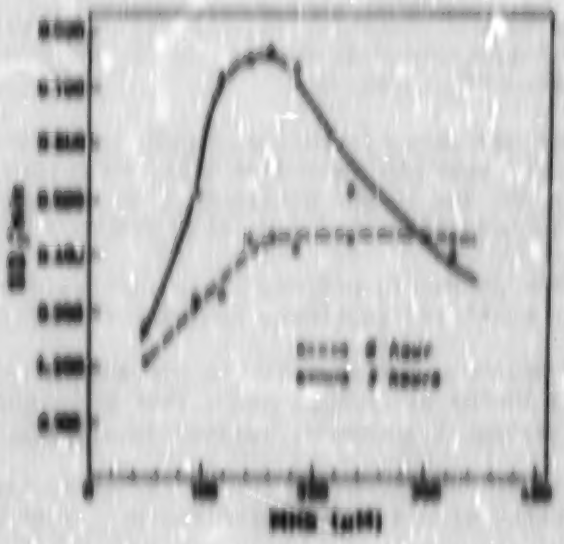


Figure XVII-3. Changes in enzyme activity after exposure to MMb. Preincubated mixtures were as described in Assay I, using buffer citrate (pH 4.7). Control assays (not illustrated) preincubated for 3 hrs. without the substrate show similar activities as those assayed immediately.

of MetHb. The maximum activity obtained at high MetHb concentration is higher at pH 7.4 than at 4.7.

When the enzyme was further purified on a sephadex G 200 column (eluente- 10 mM phosphate buffer pH 7.0) the same peak activities were obtained at pH 7.4 and 4.7.

If we estimate the intraerythrocyte Hb concentration at about 4-5 mM, the acidic maximum activity occurs when MetHb level is about 2% of the total Hb, while at pH 7.4 the maximum activity is obtained at about 50% of the total intracellular Hb. This phenomenon is compatible with our findings(10) that recovery of MetHb *in vivo* increases with MetHb concentration.

An interesting feature exhibited by the curve is the inhibitory effect of MetHb at high concentrations. This effect appears at both high and low pH but at different concentrations (Figures XVII-1 and XVII-2). Inhibition is peculiar to MetHb as the other substrates (Hb, NAD and NADH) do not affect the enzyme in a concentration range at physiological levels. Metmyoglobin, with a structure similar to that of MetHb but a quarter of its mol weight, showed the same characteristic sigmoid curve (n = 3) and the inhibitory effect at high concentration. Incubation of hemolysates in the presence of different concentrations of MetHb can convert the enzyme after the exposure to its substrate to a type more sensitive to MetHb (Figure XVII-3), both as activator and inhibitor of the system.

Sigmoid curves are common in enzymes following cooperative kinetics(9). Other interpretations that do not involve allosteric mechanisms are also possible(11).

However, our preliminary findings support the interpretation that the kinetics of the enzymatic reaction exhibits a real cooperative mechanism and is not due to the presence of an inhibitor or of improper substrate concentrations. The main findings are as follows:

1. Different enzymatic preparations, such as hemolysate and various purified fractions, exhibit sigmoidal curves.

2. MetHb reductase activity in the presence of different substrates (MetHb or metmyoglobin), from different sources and after various treatments, follows an allosteric behavior.

3. Dialysis of the enzyme yields an identical kinetic curve as the control with a cooperative factor (n) of 2.3 in both cases.

4. Controlled preincubation of the enzyme with urea in various concentrations up to 8M did not change the cooperative factor (n).

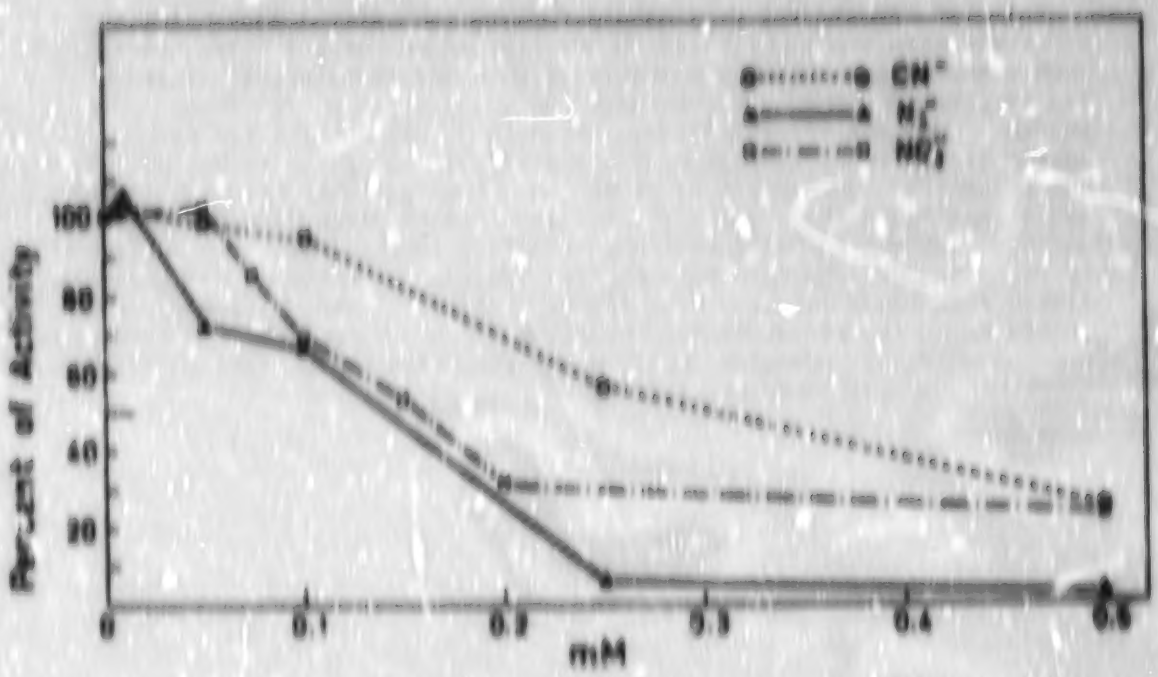


Figure XVII-4. The inhibitory effect of several unions on the methemoglobin reductase activity. The determinations were done in the presence of buffer citrate pH 4.7.

5. After fractionation of the enzyme with ammonium sulfate, the main activity was present in a wide range varying from 55-90% of saturation.

6. Controlled pre-incubation of the enzyme with trypsin increased the cooperative factor.

7. Dilution of the enzyme preparation decreased the cooperative factor.

In our laboratory, preliminary purification of the enzyme led to a protein with a mol weight of 66,000 (determined by gel filtration), double the figure reported recently by Kuma and Inomata(12). These two preparations may be the monomer and dimer types of the same enzyme. When the effect of ionic strength on enzyme activity was tested it was found that concentrations of Na^+ and K^+ up to 200 mM had no effect. The effect of anions on enzyme activity was also tested and it was found that $COOH^-$, I^-, F^-, NO_3^- had no effect but CN^-, N_3^-, and NO_2^- inhibit the enzyme either by direct reaction with the enzyme or by changing the substrate to a complex (NO_2-MetHb) which is reduced more slowly than MetHb itself. (See Figure XVII-4). Further evidence is necessary on the structure and mechanism of the enzyme. Such information will advance our knowledge on the MetHb reduction which seems to operate through a sophisticated mechanism.

REFERENCES

1. Scott, E.M. in Hereditary Disorders of Erythrocyte Metabolism (E. Beutler, ed.) pp. 103-113, Grune and Stratton, New York, 1968.

2. Scott, E.M. and McGraw, J.C., Purification and Properties of Diphosphopyridine Nucleotide Diaphorase of Human Erythrocytes, J. Biol. Chem. 237, 249-252, 1962.

3. Hegesh, E. and Avron, M., Enzymatic Reduction of Ferrihemoglobin Purification of a Ferrihemoglobin Reductase from Human Erythrocytes, Biochem. Biophys. Acta 146, 397-408, 1967.

4. Sugita, Y., Seuchi, H. and Yonoyama, Y., Purification of Reduced Pyridine - Nucleotide Dehydrogenase from Human Erythrocytes and Methemoglobin Reduction by Enzyme, J. Biol. Chem. 246, 6072-6078, 1971.

5. Hegesh, E., Gruener, N., Cohen, S., Rochkovsky, R., and Shuval, H.I. Sensitive Micromethod for Determination of Methemoglobin in Blood, Clin. Chem. Acta 30, 679-682, 1970.

6. Drabkin, D.L. and Austin, J.H., Preparations from Washed Blood Cells; Nitric Oxide Hemoglobin and Sulfhemoglobin, J. Biol. Chem. 112, 51-65, 1935.

7. Lee, H. J. and Wilson, I.B., Enzymic Parameters: Measurement of Vmax Km, Biochem. Biophys. Acta 242, 519-522, 1971.

8. Lowry, O.H., Rosebrought, N.J., Farr, A.L. and Randall, R. J., Protein Measurement With the Folin Phenol Reagent, J. Biol. Chem. 193, 265-275, 1951.

9. Atkinson, D.E., Regulation of Enzyme Activity, Ann. Rev. Biochem., 35, 85-124, 1966.

10. Gruener, N. and Shuval, H.I., in Environmental Quality and Safety (F. Coulston and F. Korte, eds.), Vol 1 pp 216-226, Georg Thein Verlag and Academic Press, Stuttgart and New York.

11. Ferdinand, W., Interpretation of Non-Hyperbolic Rate Curves for 2 - Substrate Enzymes - A possible Mechanism for Phosphofructokinase, Biochem. J., 98, 278-284, 1966.

12. Kuma, F. and Inomata, The Purification and Molecular Properties of Reduced Nicotinamide Adenine Dinucleotide - Dependent Methemoglobin Reductase, J. Biol. Chem., 247, 556-561, 1972.

SECTION XVIII

CHANGES IN THE MOTOR ACTIVITY OF MICE GIVEN SODIUM NITRITE DRINKING SOLUTION

INTRODUCTION

Induced methemoglobinemia in mice treated with sodium nitrite drinking solution causes lowering of oxygen carrying capacity of the blood. Furthermore, apart from methemoglobin production, sodium nitrite toxicity in itself interferes with several other bodily physiological mechanisms.

It is hypothesized that low levels of chronic subclinical methemoglobinemia would cause major activity deviations in mice treated with sodium nitrite drinking solution.

In this study C57bl/6J mouse was chosen for its known high activity, as well as for its homogeneous genetic constitution which would reduce the individual biological differences in the inbred mice.

The activity measured in the barrier activity box is oriented toward the environment, i.e., toward different components of the barrier box which are either arrived at by crossing the barrier, by peeking, or by grooming. The activity thereby measured is distinguishable from such undirected activity as is measured in an activity wheel. In the activity box the activity measured is more free responding than it would be in the activity wheel. In an effort to detect a correlation between the possible effects of subclinical methemoglobinemia and motor activity changes the present behavioral study was undertaken.

In the present study it was intended to investigate behavioral changes due to chronic treatment of $NaNO_2$ in C57bl/6J mice using deviations in the motor activity as the main parameter.

MATERIALS AND METHODS

A population comprised of 71 male C57bl/6J mice of 30 ± 5 days old was used in this study. Animals were divided into five groups with each group comprised of 15 mice. The five groups were as follows:

1. Group A - Control group, received only tap water.
2. Group B - Experimental group, received 1000 mg/l $NaNO_2$ in its drinking water.

3. Group C - Experimental group, received 1500 mg/l $NaNO_2$ in its drinking water.

4. Group D - Experimental group, received 1000 mg/l $NaNO_2$ in its drinking water.

5. Group E - Experimental group, received 100 mg/l $NaNO_2$ in its drinking water.

All the animals were kept in the animal room where dark and light hours as well as room temperature were controlled automatically. Room temperature varied between 19-22°C and the relative humidity varied between 45%-60%. Dark hours started at 10 A.M. and ended at 10 P.M. Water and nitrite drinking solution per 24 hours were measured and no rejection of sodium nitrite drinking solution was noticed. The mean daily intake of water and sodium nitrite drinking solution is shown in Table XVIII-1. All the experimental groups were treated with sodium nitrite drinking solution for three weeks.

The behavioral tests were carried out between 2-4 P.M., the time of highest intake of drinking solution. Our studies on chronic exposure of rats to sodium nitrite in drinking water (this report) showed that rats, being nocturnal animals, consume 80% of their water at night, and that by 10 A.M. they may no longer show significant MetHb levels even when consuming water high in nitrites. The measurements showed a peak in water consumption and MetHb level in the middle of the night. In designing this experiment the "day" and "night" hours in the animal room were adjusted so that blood examinations be taken at about the rats' "midnight", so as to detect the expected maximum daily MetHb concentration.

A barrier activity box(1) was used for the motor activity measurements. The activity box was divided into four quarters by barriers (Figure XVIII-1). After introducing a mouse to the first quarter, immediately the following parameters for motor activity measurements were observed and scored: Grooming, Peeking, and Jumping.

Activity as measures:

1. By completely crossing the barrier (Jumping);

2. Peeking over the barrier but returning to its previous enclosure.(Peeking)

3. Licking face with tongue or brushing with forelimbs.(Grooming)

Each observation lasted for five minutes, and the aforementioned parameters were scored, after which time tail blood was taken for Hb and MetHb determinations.

Table [illegible]-1. RELATIONSHIP BETWEEN WATER INTAKE IN VICE AND EXPOSURE TO $NaNO_2$

MEAN DAY'S INTAKE OF $NaNO_2$ mg/kg

Nitrite Concentration of Water mg/l

E	D	C	B
100 mg/l	1,000 mg/l	1,500 mg/l	2,000 mg/l

Nitrite Intake mg/kg

0.8	80.0	135.3	177.7

*Mean body weight = 45 gm

Mean water intake = 4.0 ml/day

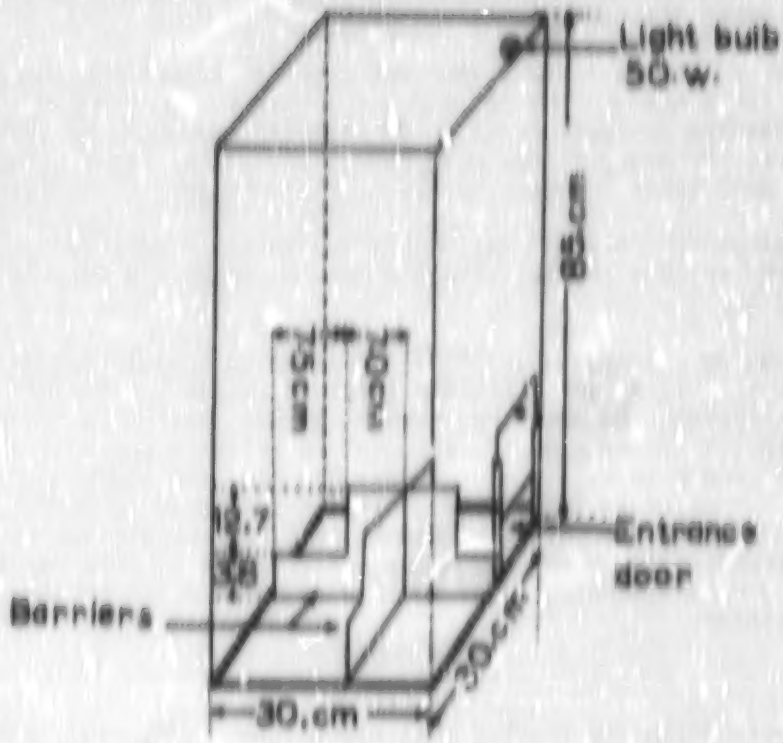


Figure XVIII-1. BARRIER ACTIVITY BOX

RESULTS

The mean Hb of the control group as well as that of the experimental groups showed to be 17.74g% with S.D. of 0.46. The mean MetHb percent in the control group was 0.5% and the mean activity was 40.57/5 min. In mice in Group B (2000 mg/l of $NaNO_2$) the mean MetHb percent was 10.1% with a mean activity of 31.0/5 min.

The results of the tests are tabulated in Table XVIII-2. Table XVIII-2 shows comparison of motor activity and the MetHb percent between the control group and experimental groups.

In Group E, 100 mg/l $NaNO_2$ does not seem to produce any change in blood MetHb level nor any change in motor activity. In Group D, 1000 mg/l $NaNO_2$ changes produced in blood MetHb level and motor activity were insignificant. In Group C 1500 mg/l $NaNO_2$, changes in blood methemoglobin begin to be noticeable but with no significant changes in the motor activity.

The pool of the obtained data was arranged in an array according to MetHb in order to show the relationship between MetHb and motor activity (Table XVIII-3).

As it can be seen in Table XVIII-3, 58% of the population fall within the range of normal methemoglobin and 15% fall within the border line, where slight increase in MetHb begins to show diminuation in motor activity. The remaining 27% showed a marked decrease in activity and an increase in the MetHb with a level of significance P=0.01. The coefficient of correlation "r" calculated from the pool of the data is -0.6480. From the obtained pool of the data the (Figure XVIII-2) coefficient of correlation was calculated for MetHb and motor activity and in this way a regression line was obtained which shows an inverse correlation between the two sets of variables.

DISCUSSION

It is noted from the results that 100 and 1000 mg/l $NaNO_2$ did not raise significantly blood methemoglobin levels nor did they change the motor activity of the mice. In mice Groups C and B, 1500, and 2000 mg/l $NaNO_2$ produced significant changes both in blood methemoglobin level as well as effect in lowering of motor activity of mice. This phenomenon was also observed in rats treated with sodium nitrite drinking solution.

The regression line shows an inverse correlation between the two sets of variables with P=0.002. However, when an attempt was made to correlate the motor activity and the calculated mean sodium nitrite dose mg/kg B.W., only a poor correlation between these two variables was indicated. The most probable explanation of this is that the true sodium nitrite intake between the animals varied greatly.

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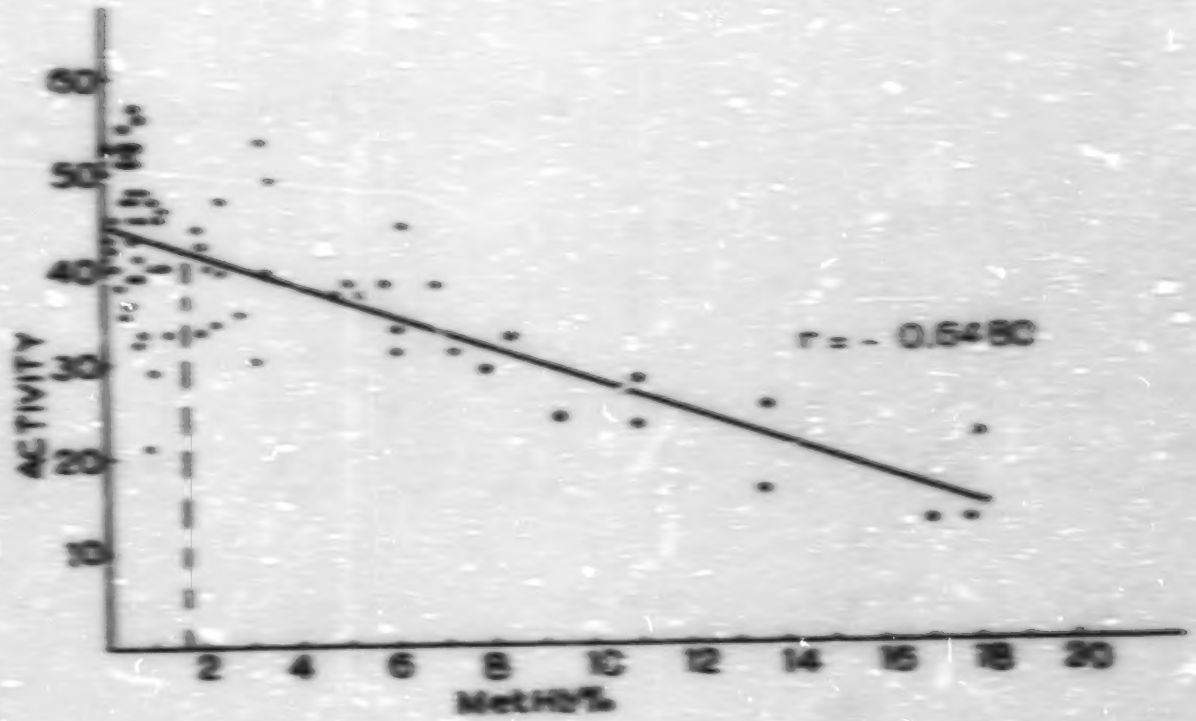

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Table XVIII-3. RELATION BETWEEN METHEMOGLOBIN AND MOTOR ACTIVITY IN WEEP CONSUMING $NaNO_2$ IN DRINKING WATER

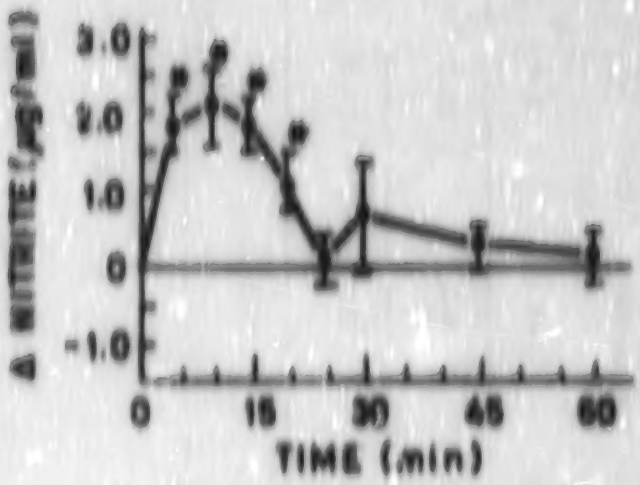
Metab %	$\bar{X}$ Activity	n	p
0.0 — 1.9	45.8	41.0	
			>0.05
2.0 — 5.9	40.9	22.0	
			<0.02
4.0 — 7.9	35.7	10.0	
			<0.01
8.0 — 13.6	27.5	9.0	

r = -0.6480 Σn = 72.3

p = level of significance.

FIG. 3

MEAN DIFFERENCE BETWEEN EXPERIMENTAL AND CONTROL ANIMALS (CONTROL MINUS EXPERIMENTAL) IN NITRITE LEVELS AT EACH TIME POINT STUDIES



* *statistical significance* $p \leq 0.05$
shown are means ± *S.D*

Figure XVIII-3. MEAN DIFFERENCE BETWEEN EXPERIMENTAL AND CONTROL ANIMALS (CONTROL MINUS EXPERIMENTAL) IN NITRITE LEVELS AT EACH TIME POINT STUDIES

The threshold of lowering of motor activity in C_{57} Mice starts at less than 2% MetHb. This is the upper limit of methemoglobin which is found in normal infants (this report).

There are a number of factors which may as well participate in the lowering of motor activity of mice which should be taken into consideration. Sodium nitrite can cause the conversion of myoglobin into metmyoglobin which interferes with muscle activity by lowering of the oxygen capacity of the muscular tissues. Nitrites are also known to be anticholinergic substance. Nitrites can also act directly on the central nervous system.

In the study of Petukov & Ivanov(2) the mean MetHb of children aged 12-14 years exposed to high levels of nitrates in drinking water (105 ppm as NO_3) is reported to be 5.3% which indicates that low levels of chronic subclinical methemoglobinemia may eventually lead to bodily physiological changes. This level is reported to bring about some changes in psychophysiological reactions of these children.

It is as yet premature to extrapolate the results of these studies as to the significance of sodium nitrite on human health and behavior. However, since sodium nitrite is such a widely used food additive and nitrates are so commonly found in water and certain vegetables, it is essential that the implications of these environmental exposures be carefully followed up and studied.

REFERENCES

1. Guttman, R., Lieblich, J., and Naftali, G. Sequential Behaviour in a Novel Situation in Two Inbred Strains of Mice and Their Hybrids. Life Sciences, Vol. 8, Part II, pp. 597-600, 1969.

2. Petukov, N.I. and Ivanov, A.V. Investigation of Certain Psychophysiological Reactions in Children Suffering from Methemoglobinemia due to Nitrate in Water. Hyg. San. U.S.S.R. 35, 29-31, 1970.

SECTION XIX

THE EFFECT OF NITRITES ON ISOLATION-INDUCED AGGRESSION IN MICE

The interest in the effect of environmental agents on behavior is on the increase as a growing segment of the population is exposed to such agents. Nitrates and nitrites are frequently ingested in drinking water, natural food such as vegetables, and in processed foods when food additives have been utilized. One of the complications following excessive nitrate and nitrite intake is methemoglobinemia. This disease is characterized by decrease in the oxygen transport capacity of the blood. Nitrite which is the direct toxic agent can oxidize hemoglobin to methemoglobin which is biologically inactive. Nitrates can be reduced to nitrites mainly by microbiological activity in the food or in digestive systems. Infants in the first months of life are especially susceptible to methemoglobinemia from these salts(1).

The question of the health implications at the subclinical level of exposure to nitrates and nitrites is of considerable importance, because detection of symptoms at such levels is difficult and thus no preventive actions are likely to be taken.

We have already shown(2) that C_{57}-Bl mice that have been given sodium nitrite in their drinking water, showed an increase in methemoglobin level. The average was 1.2% at a nitrite concentration of 1 g/l (control mean level = 0.6%) and 10.1% of methemoglobin at 3 g/l of nitrites. Levels of 10% or higher of methemoglobin are considered clinically significant. In the same report we have shown that nitrites reduced the motor activity of the mice but only at the highest concentration (3 g/l).

In the following section, we will show that nitrites cause a significant increase in the aggression of mice.

METHOD

Ten C_{57}-Bl female mice were mated with males of the same strain. Each couple was placed in a separate cage. Five couples were given a sodium nitrite solution (1 g/l in tap water) for their drinking water and the other five couples drank tap water. At birth, the adult males were removed from the cages and the newborne mice were kept with their mothers for the next 21 days. The mothers who drank $NaNO_2$ during gestation continued to receive the same solution through the nursing period.

The young were weighed twice a week through the nursing period. At weaning (after 21 days) twelve young males from each group were randomly chosen and each was placed in a separate cage and isolated for eight weeks. The nitrite group continued to get the nitrites at the same level (1 g/l) throughout the isolation period, and for the first five weeks of the behavioral tests.

At the end of the isolation period the mice were tested for isolated-induced aggression by a modified method of Banerjee(3). Each confrontation between two mice was allowed to take place for ten minutes in a square fighting cage, and behavior was rated according to a predetermined aggression scale with maximum score of 18.

Each animal was exposed to another animal once a week, six times, four of the six sessions were intra-group confrontations and two were extra group confrontations. There was no set order for these confrontations because the order might influence the results of the confrontations.

After five weeks the experimental animals were taken off the nitrite solution and were given tap water to drink. After a break of two weeks the tests were resumed and the first four sessions were repeated. During the latter period all the animals drank tap water.

RESULTS AND DISCUSSION

The results of the confrontation sessions are presented in Table XIX-1 and XIX-2. The cumulative score for each group was averaged per the number of the sessions. Table XIX-1 shows that there was an increase in the aggressive behavior of the treated group particularly when this group met the controls. At these sessions, the controls had a significantly low score. Returning the experimental group to regular tap water was accompanied by a decrease in aggressions to the level of the control group which did not change during this time. The simultaneous increase in the mean score of the water group when exposed to the previous nitrite group shows that the score for the individual in each session is not independent of its opponent.

Table XIX-1. THE EFFECT OF SODIUM NITRITE ON AGGRESSION IN MICE

Group	Number of Confrontations	Mean Aggression Score	S.D.	p*
1. H_2O - intra	44	9.01	3.14	-
2. H_2O - extra	23	5.65	3.55	<0.01
3. $NaNO_2$ (1g/l) -intra	36	10.55	4.68	<0.05
4. $NaNO_2$ (1g/l) -extra	27	12.05	3.66	<0.01

*The differences in the mean aggression score between Group 1 and Groups 2, 3, and 4, are analyzed statistically according to the "t" test.

"Intra" means sessions in which mice exposed to others belong to the same experimental group. "Extra" means sessions in which mice exposed to others belong to the other experimental group.

Table XIX-9. AGGRESSION OF MICE AFTER RETURNING TO TAP WATER

Group	Number of Confrontations	Mean Aggression Score	S.D.	p*
1. H_2O - intra	32	8.97	5.00	-
2. H_2O - extra	15	8.77	5.00	N.S.
3. $NaNO_2$ (1g/l)-intra	38	9.43	5.31	N.S.
4. $NaNO_2$ (1g/l)-extra	15	9.50	5.20	N.S.

*The differences in the mean aggression score between Group 1 and Groups 2, 3, and 4, are analysed statistically according to the "t" test.

"Intra" means sessions in which mice exposed to others belong to the same experimental group. "Extra" means sessions in which mice exposed to others belong to the other experimental group.

Several reports have recently described the different phenomena caused by nitrites or nitrates on the central nervous system(4,5,6). However, no suggestion has been given regarding the site(s) or mode of action of these ions in the tissue. This study hints that at least the behavioral effect is reversible and disappears after a short break. Elucidation of the mechanism of this aggression phenomenon might help in the evaluation of the risks of exposure to nitrates and nitrites.

REFERENCES

1. Gruener, N., and Shuval, H.I. "Health Aspects of Nitrate in Drinking Water," in Water Quality Research, Ann Arbor, Humphrey Science Publishers, Ann Arbor-London, pp 89-105 1970.

2. Behrouzi, E., Guttman, R., Gruener, N. and Shuval, H.I. Changes in Motor Activity of Mice Given Sodium Nitrite in Drinking Solution, Israel J. Med. Sci. 8, 1007, 1972.

3. Banerjee, U. An Inquiry into Genesis of Aggression in Mice Induced by Isolation, Behavior XL, 66-69, 1971.

4. Robinson, S., Behrouzi, E., Gruener, N., and Shuval, H.I. The Effect of Chronic Exposure to Sodium Nitrite on the Electroencephalogram of Rats, Environmental Research 5, 4, 1972.

5. Petukov, N.I. and Ivanov, A.V. Investigation of Certain Psychophysiological Reactions in Children Suffering from Methemoglobinemia Due to Nitrate in Water, Hyg. San. 35:39-41, 1970.

6. Henderson, R.R., and Raskin, H.H. "Hot-Dog," Headache: Individual Susceptibility to Nitrite. Lancet, December, 1142, 1972.

SECTION XX

THE EFFECT OF CHRONIC EXPOSURE TO SODIUM NITRITE ON THE ELECTRO ENCEPHALOGRAM OF RATS

INTRODUCTION

In the course of our analysis of the possible side effects of raised $NaNO_3$ levels we considered the influence of reduced oxygen supply on sensitive organs such as the brain as a potential line of investigation. As yet, little attention has been paid to the toxicological effects of nitrites on the central nervous system. Petukov and Ivanov(1) have recently reported on psychophysiological changes in school children in regions of high nitrates concentrations in drinking water.

The objectives of the present study were to investigate the long-term chronic effects of $NaNO_2$ administered in drinking water on the CNS and changes which it would cause in the brain electrical activity as indicated by EEG deviations. Two sets of experiments were carried out: the first by Dr. S. Robinson and Mr. E. Behrens and the second by Dr. M. Gutnick and Mr. M. Dalit.

FIRST EXPERIMENT

METHODS AND MATERIALS

Sixteen male albino rats of the Hebrew University's "Sabra" strain weighing initially 200±10 gm each were used. Four monopolar ball-pointed silver electrodes were stereotactically placed on the dura mater of the cortex in the left and right anterior and left and right posterior areas(2). The electrodes were fixed in place with dental cement and later joined to a connector, cushioned on the skull. Following the implantation of the electrodes, the animals were allowed to recuperate for two to three weeks before the recordings were made.

Animals were divided into four groups, each group comprised of four rats. Each rat was placed in a separate cage. All groups received plain tap water for a period of two weeks during which time recordings from all of them were taken every three days in order to serve as controls of their own for later comparisons. The three experimental groups received sodium nitrite in their drinking water for a period of two months, after which time the $NaNO_2$ was removed from their drinking water and the animals received plain tap water for an additional period up to four and a half months. The four groups were as follows:

Group A - Control Group received only plain tap water
Group B - Experimental Group received: 3000 mg/l $NaNO_2$
Group C - Experimental Group received: 300 mg/l $NaNO_2$
Group D - Experimental Group received: 100 mg/l $NaNO_2$

All the recordings were made under identical conditions and time. The room temperature varied between 19° and 22° C. During the recordings the animals were kept in a round metallic cage and permitted to move about freely while recordings were made and animals' behavior could be observed. Following connection of the EEG adapter to the socket, the animals were allowed a period of 15-20 minutes for adjustment. An Alvar 8 channel electroencephalograph was used in this study. After each recording blood samples were taken from the tail of the rats for hemoglobin and methemoglobin determination. The evaluation of EEG records was made by visual methods and wave group counting.

RESULTS

Different concentrations of $NaNO_2$ in the drinking water appear to reveal characteristic changes in the EEG pattern as well as methemoglobin formation and behavioral changes.

Group A

Group A showed no changes in the brain electrical activity or in the behavior during the three and a half months period of follow-up. There were also no changes in the MetHb percent. The mean MetHb percent was 0.5% and varied between 0.0 and 0.8% which is normal in rats (See Figure XX-1).

Group B

This group was exposed to 2000 mg/l $NaNO_2$ drinking solution which is equivalent to a daily dose of 280 mg/kg body weight of the rat per twenty-four hours. What appear to be changes in the brain electrical activity were noted from the fourth day of treatment. There appeared diffused spikes and sharp waves and the frequency of the background electrical activity gradually increased from E.F.8.4 c/s to E.F.11.5 c/s* (See Table XX-1), achieving maximum of frequency after two weeks of treatment. After two weeks, general paroxysmal outbursts of high amplitude, up to 240 uv sharp waves became apparent mixed with diffused theta waves (See Figures XX-2 and XX-3).

During the two months of chronic exposure to $NaNO_2$ the EEG records were of similar pattern. MetHb percent ranges varied between 6.7 and 30.8% with average 13.16%. All the rats in this group were generally sedate when compared with the control group as well as with their own behavior before the treatment.

*(E.F. c/s = mean frequency cycles/second)

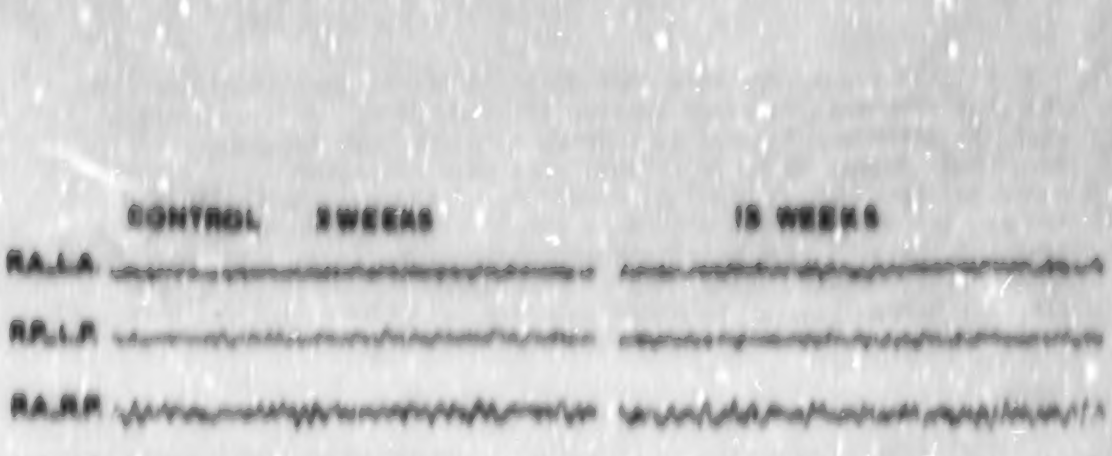


Figure XX-1. THE EEG OF THE CONTROL GROUP REMAINED UNCHANGED DURING THE THREE AND A HALF MONTHS OF FOLLOW-UP PERIOD. (Group A Control)

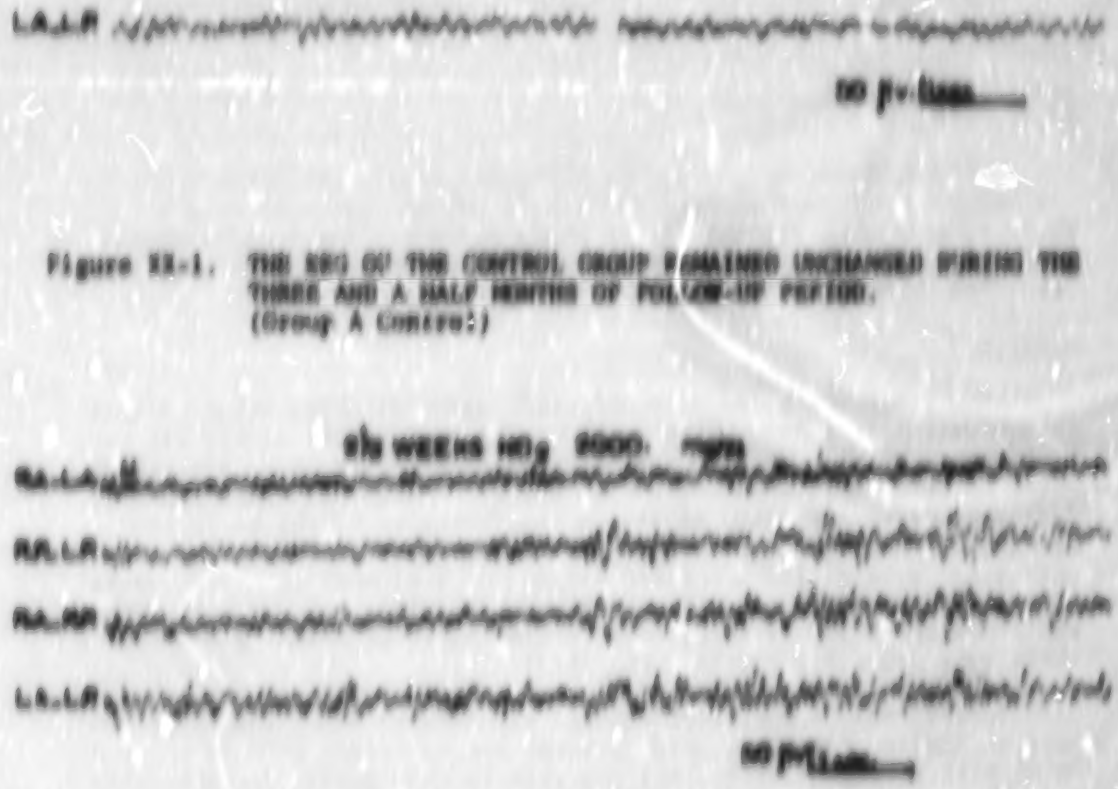


Figure XX-2. CHANGE IN BACKGROUND ACTIVITY AFTER TWO AND A HALF WEEKS OF $NaNO_2$ DRINKING SHOWING ACCELERATION OF THE RHYTHM, APPEARANCE OF SPIKES AND SHARP WAVES MIXED WITH DIFFUSED THETA AND DELTA WAVES. (Group B)

Table XX-1. RELATIONSHIP BETWEEN MEAN FREQUENCY OF BACKGROUND BRAIN ELECTRICAL ACTIVITY, AMPLITUDE RANGES AND METHEMOGLOBINEMIA IN RATS CONSUMING DIFFERENT CONCENTRATIONS OF $NaNO_3$

	Group A Tap Water	Group B $NaNO_3$ 5000 mg/l	Group C $NaNO_3$ 500 mg/l	Group D $NaNO_3$ 100 mg/l
EEG $\bar{X}$f c/s	8.4	8.4	8.4	8.4
Prior to Exposure S.D.	1.48	1.48	1.48	1.48
EEG Amp. Range µV Prior to exposure	30-60	30-60	30-60	30-60
$\bar{X}$ Metab percent	0.5	0.5	0.5	0.5
Prior to Exposure S.D.	0.24	0.24	0.24	0.24
EEG $\bar{X}$f c/s		11.5	8.5	8.1
During Exposure S.D.		1.11	0.42	0.75
EEG Amp. Range µV During Exposure		50-240	50-200	30-170
$\bar{X}$ Metab percent		12.2	3.0	1.1
During Exposure S.D.		5.63	0.94	0.47
EEG $\bar{X}$f c/s		8.2	8.0	7.0
Return to tap water S.D.		0.53	1.17	0.91
EEG. Amp. µV Return to tap water		50-240	50-170	30-80
$\bar{X}$ Metab percent	0.49	0.70	0.47	0.43
Return to tap water S.D.		0.53	0.17	0.10

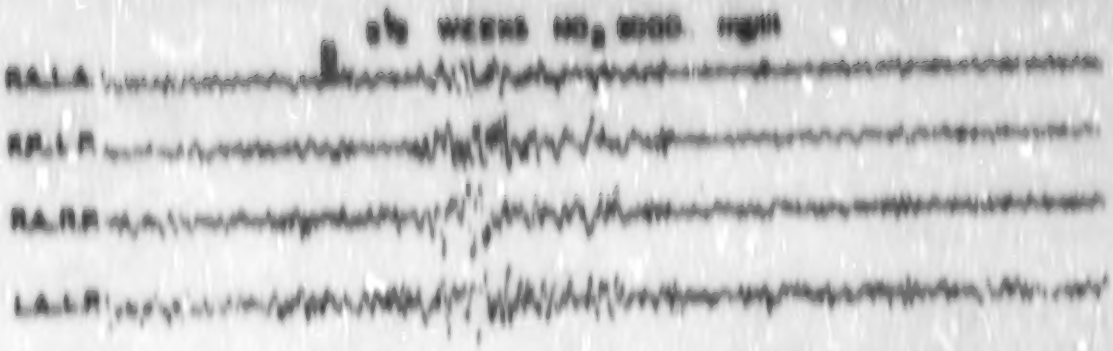


Figure XX-3. APPEARANCE OF GENERAL PAROXYSMAL OUTBURST OF HIGH VOLTAGE, THETA, DELTA AND SHARP WAVES. (Group B).

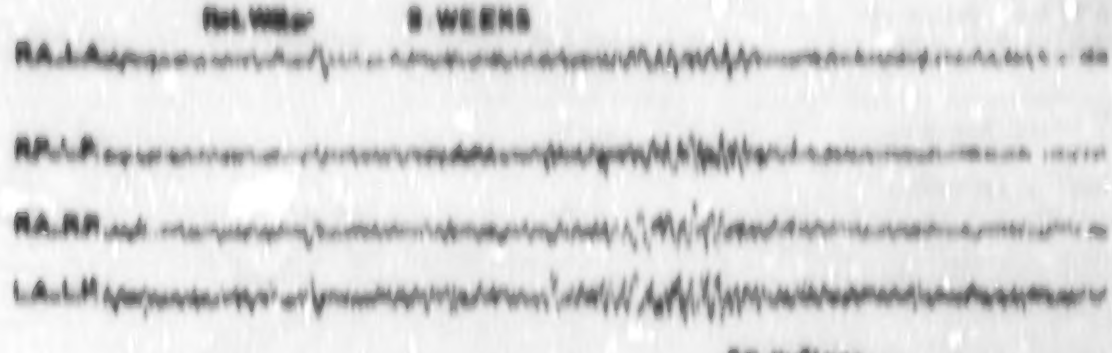


Figure XX-4. BACKGROUND ACTIVITY STILL RAPID WITH GENERAL PAROXYSMAL OUTBURSTS OF HIGH VOLTAGE DELTA, THETA AND SHARP WAVES. (Group B).

During the electrical outburst their behavior suddenly changed and the rats remained motionless. After the end of the outburst their motor activity returned to that of their previous pattern. During the electrical outbursts, there were no clinical convulsions or any collapse and the animals were thought to be awake. To check this a sharp noise was made during a few such motionless periods. The animals were alert to the noise but the outburst continued unabated.

Two of the four rats in the group showed pronounced cyanosis characteristics of methemoglobinemia with MetHb percent up to 30% and they seemed more sedate than the rest. In these rats the frequency of the occurrence of paroxysmal outbursts was greater and also of longer duration.

During the four and a half months of follow-up after withdrawal of $NaNO_2$ the EEG pattern was similar to that during the $NaNO_2$ treatment, i.e., the outbursts continued to appear and the background activity had a 5.7-9.2 c/s and while the MetHb percent returned to normal and was between 0.5% and 0.7% (See Figure XX-4).

Group C

This group was exposed to 300 mg/l $NaNO_2$ drinking solution from which was received an equivalent daily dose of 42 mg/kg body weight of the rats per twenty-four hours. Here also changes appeared after four days of $NaNO_2$ treatment and the EEG recordings showed spikes and sharp waves, similar to Group B. The background activity appeared to be slower than that of their own control.

Here too, after two weeks of treatment, there appeared general paroxysmal outbursts comprising slow and sharp waves with predominant slow waves of theta and delta bands. The frequency of the occurrence of these outbursts was less than in Group B, but the amplitudes were similar (See Figure XX-5).

During the follow-up of three and a half months after the withdrawal of $NaNO_2$, the EEG background activity had a 5.7-8.0 c/s. General outbursts still continued to appear. (See Figure 6).

The MetHb percent during the treatment ranged between 1.7 and 4.5% and after the withdrawal of $NaNO_2$, it ranged between 0.5 and 0.7%. During the paroxysmal outbursts the animals in Group C behaved similarly to those in Group B.

Group D

This group was exposed to 100 mg/l $NaNO_2$ drinking solution from which was received an equivalent daily dose of 14 mg/kg body weight of the rat per twenty-four hours. Here too, what appear to be slight changes in the EEG were noted after four days, and they seemed to be similar to those described in Group C, except that the frequency of the occurrence of outbursts was lower.

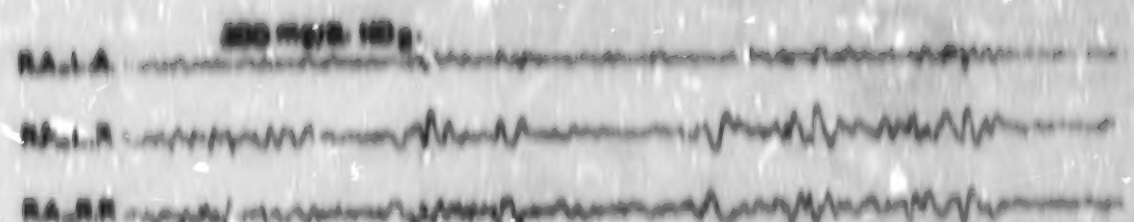


Figure XX-5. APPEARANCE OF SLOWED BACKGROUND ACTIVITY, DIFFUSED THETA AND DELTA WAVES AFTER TWO WEEKS OF $NaNO_2$ DRINKING. (Group C)

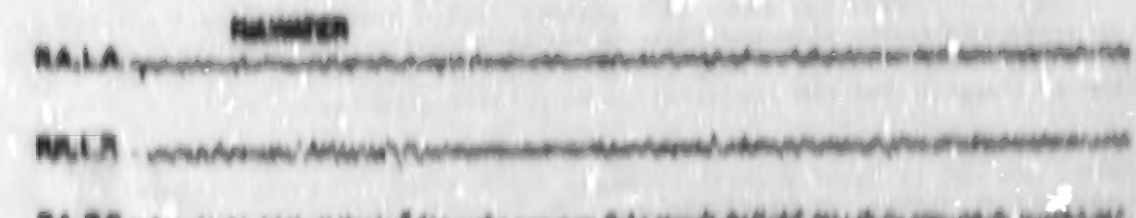


Figure XX-6. BACKGROUND ACTIVITY IS STILL SLOWER THAN THE CONTROL AND OFF-BURSTS CONTINUE TO APPEAR. (Group C).

During the return to plain tap water for a period of two and a half months, slow background activity continued 8.f. c/s 7.0. The general outbursts disappeared after two weeks but spikes and sharp waves were still recorded. The MetHb percent ranged between 0.5 and 2.4% (2 MetHb percent 1.1) and after the withdrawal of $NaNO_2$ the MetHb percent ranged between 0.3 and 0.6% (See Figure XX-8).

In general, their behavior was similar to Group A, but during the general outbursts they were similar to Group B.

DISCUSSION

The results indicate that $NaNO_2$ may have some effect on the EEG of the rats treated chronically with this substance. In the unfolding of the recordings obtained, it is noted that increased levels of $NaNO_2$ in drinking water appear to lead to:

1) more accentuated changes in the EEG;
2) increase in MetHb percent;
3) decrease in general motor activity as observed visually.

Changes appeared in the background EEG at the three concentration levels. In rats exposed to 2000 mg/l $NaNO_2$, the background brain electrical activity appeared to be faster than the control group as well as the other two experimental groups, while at 500 mg/l and 100 mg/l $NaNO_2$, the background electrical activity became slightly slower than the control group. Spikes and sharp waves appeared in the EEG of all the rats in the experimental groups from the fourth day of the treatment and continued on during the whole length of the experiment as well as after their return to plain water. It might be hypothesized that the described EEG changes might be due to brain anoxia caused by degenerative vascular changes in the brain. Huper and Lendsburg(3) report on the brain vascular degenerative changes and vacolation in midbrain and brainstem with erythroltetranitrates and $NaNO_2$ and relate the afore-mentioned phenomena to stagnant hyperemia and hyperemia caused by vasodilatory effects of $NaNO_2$.

Another possible cause of hypoxia might be due to nitrite inducing methemoglobinemia which results in the lowering of oxygen carrying capacity of the blood. Garbus(4) in a brief communication has reported the irreversible acceleration of the EEG rhythm in rabbits acutely treated with $NaNO_2$ and related this phenomenon to anoxic hypoxia which is caused by methemoglobinemia.

In our present investigation features which might suggest brain hypoxia expressed by the appearance of slowed background brain electrical activity in EEG were seen mainly at low concentrations of $NaNO_2$ in the drinking water of the rats with the mean of 1.1% MetHb in Group D and 5.0% MetHb in Group C. Such levels are normal or nearly normal for

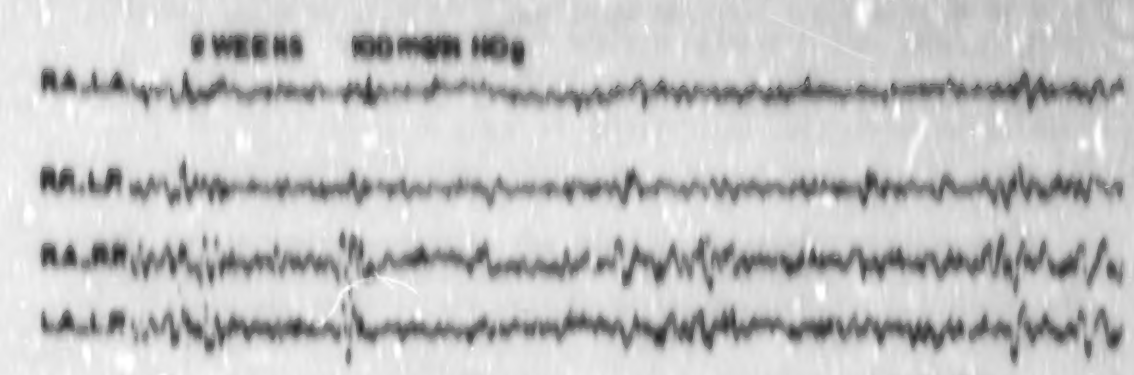


Figure XX-7. BACKGROUND ACTIVITY IS SLOWER THAN THE CONTROL WITH APPEARANCE OF GENERAL OFFBURSTS OF HIGH VOLTAGE DELTA, THETA SPIKES AND SHARP WAVES. (Group D).

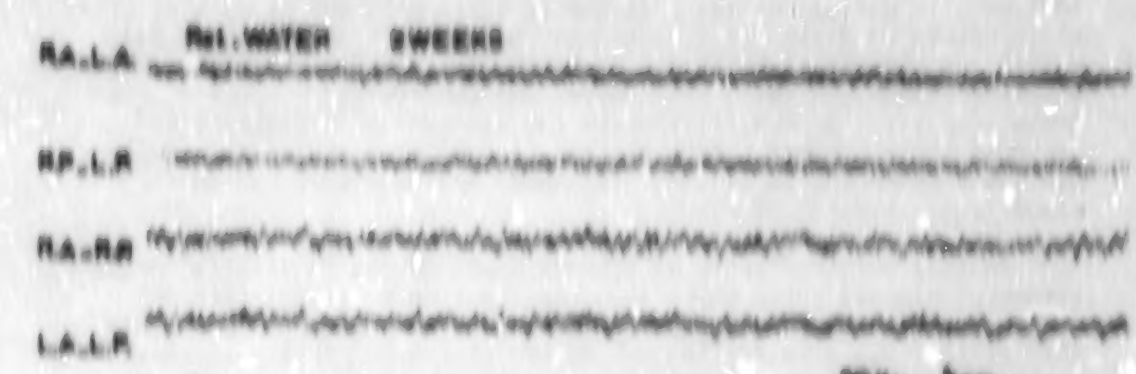


Figure XX-8. SLOW BACKGROUND ACTIVITY STILL CONTINUES WITH DIFFUSED SPIKES AND SHARP WAVES. (Group D).

rate and hypoxia would not be anticipated. It is noted that at the highest $NaNO_2$ dose of 2000 mg/l, when high Metth percent levels were detected and anoxia might logically be anticipated, the EEG showed rapid background activity not typical for hypoxic conditions.

Our findings raise the possibility that the disturbed EEG in our experimental groups after exposure to $NaNO_2$ may also be due to toxic effects of the $NaNO_2$ itself on the CNS, even at relatively low levels of exposure. During the paroxysmal outbursts it is noted that the outbursts are diffused, symmetrical, synchronised and uniform. This would seem to suggest the outbursts are of center-encephalic origin. It is seen that $NaNO_2$ at different concentrations has different characteristic effects on the EEG. This might be due to biphasic effects of $NaNO_2$ on the EEG of the rats. Such phenomena is known from the effects of other drugs such as pentobarbital.

The observed changes in the brain electrical activity of the rats appeared to be irreversible in that paroxysmal outbursts in Groups B and C, and spikes in Group D continue to appear even after the removal of $NaNO_2$ from the drinking water of the rats.

In these experiments interpretation of the EEG charts were carried out by the researchers themselves who were aware of the nitrite doses administered to the rats.

SECOND EXPERIMENT

On the completion of the first small-scale study, it was decided to continue this line of investigation but due to changes in personnel the first efforts were devoted to a recheck of the findings of the first experiment, while the first study was carried out at the Talbieh Psychiatric Hospital using their facilities and equipment under the supervision of Dr. S. Robinson, the second series was carried out in the Environmental Health Laboratory using a Grass electroencephalograph on loan from the Department of Neurology of the Hebrew University-Hadassah Hospital. The work here was supervised by Dr. Michael Gutnick, a neurobiologist from the Department of Zoology. In the second study, rats were exposed to the following levels of nitrites in drinking water with four animals per group:

a) 50 mg/l
b) 100 mg/l
c) 500 mg/l
d) 2,000 mg/l

As in the first experiment, Hebrew University Sabra male albino rats weighing between 150-230 gms had electrodes implanted on the dura mater of the cortex. After the implantation of the electrodes, the animals were given a two-week recovery period. On the completion of the

recovery period, control recordings were made on each individual rat in order to determine whether the implanted electrodes were satisfactory and whether the rats' EEG recordings were within what might be expected to be normal, predictable ranges for such animals. Four pre-exposure recordings were made at intervals of three to four days. Each recording session was of thirty minutes duration. On the completion of the two-week control period, the animals were exposed to various concentrations of nitrites in their drinking water. The concentration of the nitrites in the drinking water was checked regularly.

Three or four days after exposure to nitrites in drinking water was initiated, EEG recordings were made. These recordings were carried out at regular intervals according to a carefully standardized protocol during a period of a month and in such a way that each animal had between six and eight recordings made. After each recording session, a sample of tail-blood was taken in order to determine the level of Hetib. The recordings were made on freely-moving animals held in either grooved metal cages or in a cage made of Perspex. During the complete period of the recording, the animal was observed for unusual behavior patterns which were noted on an appropriate point on the EEG charts. Environmental conditions during each recording session were kept as uniform as possible with constant temperature and light conditions.

RESULTS

The results of the EEG recordings after the exposure to various doses of nitrites were compared with each animal's own pre-exposure EEG recording. No obvious differences could be observed in the frequency or in the appearance of unusual wave-forms or outbursts. The readings of the EEG recordings were made by three trained observers independently, two of whom were not informed of the exposure patterns of the animals whose recordings they were evaluating. No behavioral differences were noted during the recordings or at other periods, regardless of the nitrite dose to which the animals were exposed. The methemoglobin level of the rats were similar to those exposed to the same nitrite dose in the first experiment.

CONCLUSION

In the attempt to repeat the first experiment, no electrical brain-wave activity changes were detected between pre-exposure controls and post-exposure recordings, even with nitrite doses as high as 2,000 mg/l, as were found in the first study. Since the repeat-experiment also used only a very limited number of animals, it would be difficult to conclude that these findings alone totally negate the previous findings, despite the fact that the second study was carried out under more carefully controlled conditions and the recordings were evaluated independently by

trained observers who were not aware of the treatment given to the animals. At most, we can say that these conflicting findings leave us in a position where we can say little at this time as to the validity of the findings of suspected neurological changes resulting from exposure to nitrites in drinking water. Our own conflicting results and the findings of Russian researchers(4) in this area nevertheless leave this question open for further study.

REFERENCES

1. Petuknov, N.S. and Ivanov, A.V. Investigation of Certain Psycho-physiological Reactions in Children Suffering from Methemoglobinemia Due to Nitrates in Water, Hyg. Sen. 35, 29-31, 1970.

2. Nir, I., Behroozi, E., Assael, M., and Ivriani, I. Changes in the Electrical Activity of the Brain Following Pinealectomy, Neuroendocrinology, 4:122-127, 1969.

3. Hueper, W.C. and Landsberg, J.W. Experimental Studies in Cardiovascular Pathology, I. Pathologic Changes in the Organs of Rats Produced by Chronic Nitrite Poisoning, Arch. of Pathology, Vol. 29, pp. 633-648, 1940.

4. Garbus, A.M. Functional State of the Central Nervous System in "Symptomless" Methemoglobinemia, Communication in Brief, Gig. Sanit. 36(1), pp. 101-102, U.S.S.R. 1971.

APPENDIX I

SURVEY ON METHAEMOGLOBINAEMIA

QUESTIONNAIRE

Questionnaire No.:
Identity Card No.:
Date of completing form:

A. DETAILS OF INFANT

1. Surname
2. First Names
3. Sex
4. Hospital where born
5. Date of birth in kg.
6. Home address of parents
7. Name of Clinic
8. Age in months
9. Present weight

B. DETAILS OF PARENTS

10. First Name - Father: Mother:
11. Country of Birth - Father: Mother:
12. Country of Birth of Father's Parents: Father: Mother:
13. Religion

C. GENERAL

14. Was the baby away from the above-mentioned address last week?
    Yes..... No.....
15. If so, how many days ago did he return home?
16. How many days was he away from home?

    Where was he?

D. SICKNESS AND TREATMENT

17. Does the baby have diarrhea at present? Yes..... No.....
18. If so, for how many days?
19. If not, did he have diarrhea during the last month? Yes....No....
20. Was the diarrhea heavy or mild? Heavy..... Mild.....
21. Was a clinical diagnosis made? Yes..... No.....
    If so, what was the diagnosis?
22. Other illnesses over the last month:
23. Did he ever receive iron? Yes..... No.....
    If so, when?

E. NUTRITION (in the past 24 hours)

24. Mother's Milk Yes..... No.....
25. Milk Powder Yes..... No.....
    From when?
26. Pasteurized Milk Yes..... No.....
    From when?
27. Sterilized Milk Yes..... No.....
    From when?
28. Fresh Cow's Milk Yes..... No.....
    From when?
29. Goat's Milk Yes..... No.....
    Type of Milk Powder (brand name).....
    Additional comments on nutrition:.....
    .....
30. Was supplementary water given? Yes..... No.....
    If so, in what form was the additional water given?
    (e.g., water added to milk powder, or sweetened water as liquid supplement)
    (daily portions)

Other Additions to Nutrition

31. Soup Yes..... No.....
32. Porridge Yes..... No.....
33. Sausage Yes..... No.....

34. Spinach Yes..... No.....
35. Vitamin C Yes..... No.....
36. Tomatoes Yes..... No.....
37. Citrus Yes..... No.....

Clinical symptoms if any .....

APPENDIX II

List of Publications

1. Gruener, N. and Shuval, H.I., "Health Aspects of Nitrates in Drinking Water" in Developments in Water Quality Research, Ann Arbor-Humphrey Science Publishers, Ann Arbor - London, pp. 89-106, 1970.

2. Hegesh, E., Gruener, N., Cohen, S., Bucherzky, E., and Shuval, H.I. "A Sensitive Micro-Method for Determination of Methemoglobin in Blood," Clin. Chim. Acta, 30, 579, 1970.

3. Shechter, H., Gruener, N. and Shuval, H.I., "A Micro-Method for the Determination of Nitrite in Blood," Anal. Chim. Acta 60, 95, 1972.

4. Shuval, H.I. and Gruener, N., "Epidemiological and Toxicological Aspects of Nitrates and Nitrites in the Environment," Am. J. Pub. Health, 62, 1045, 1972.

5. Behrouzi, E., Robinson, S., Gruener, N. and Shuval, H.I., "The Effect of Chronic Exposure to Sodium Nitrite on the Electroencephalogram of Rats," Environ. Research 5, 4, 1972.

6. Robinson, S., Behrouzi, E., Gruener, N. and Shuval, H.I., "Changes in the Electrical Activity of the Brain of Rats Fed with Sodium Nitrite," Abstract: Proc. Symposium on Environmental Physiology, Israel J. Med. Sci., 8:1006, 1972.

7. Gruener, N. and Shuval, H.I., "Studies on the Toxicology of Nitrites," in the Environmental Toxicology and Safety, Ed. F.G. Coulston and F. Korte, Academic Press, London-New York, Vol. II, 1972.

8. Gruener, N., Shuval, H.I., Behrouzi, E., Cohen, S. and Schechter, H., "Methemoglobinemia Induced by Transplacental Passage of Nitrites to Rats" Abstract: Int.J. Agr. Res. 1972. Also Bull. Env. Cont. Toxicol. 9,44,1973.

9. Shuval, H.I. and Gruener, N., "The Association Between Nitrates in Drinking Water and Infant Methemoglobin in Several Communities in Israel," Abstract: Int. J. Agr. Res., 1973.

10. Behrouzi, E., Gottman, E., Gruener, N. and Shuval, H.I., "Changes in the Motor Activity of Mice Given Sodium Nitrite in Drinking Solution," Abstract: Proc. Symposium on Environmental Physiology, Beersheba, Israel J. Med. Sci. 8:0807, 1972.

11. Gruener, N. and Shuval, H.I., "Health Aspects of the Exposure to Nitrates in the Environment, Harefuah, July 1973.

13. Gruener, N., Varon, R. and Shuval, H.I., "Histological Changes in Rat Heart Arteries After Chronic Administration of Nitrates and Nitrites," Abstract: Proc. European Symp. on Impact of Ecological Factors on Peripheral Vascular Disease, September, 1975.

14. Shuval, H.I. and Gruener, N., "The Effects on Man and Animals of Ingesting Nitrates and Nitrites in Water and Food" in "Effects of Agricultural Isotope Studies," Proc. Joint FAO/Joint FAO/IAEA Panel Experts, Vienna, 117-130, 1974.

14. Gruener, N. and Cohen, S., "The Reduction of Methemoglobin in the Human Erythrocyte," Physiol. Chem. & Physics 5:375, 1973.

15. Gruener, N., "The Effect of Nitrites on Isolation-Induced Agression in Mice," Pharmacol. Biochem. Behavior 2:267, 1974.

16. Gruener, N., Scheuermann, D. and Shuval, H.I., "Survey of Liquid Intake in Infants," Environ. Physiol. Biochem. 4, 1974.

17. Gruener, N. and Shuval, H.I., "Evaluation of the Health Effects of Nitrates in Water" in Recent Advances in the Assessment of the Health Effects of Environmental Pollution, 1974, Commission on European Communities, Luxembourg, 2:1067-1073, 1975.

18. Gruener, N. and Tsoglin, A., "The Effect of Changes in Nitrate Concentration in Drinking Water on Methemoglobin Levels in Infants," Int. J. Environ. Studies, 7(3) 1975,(ABCD 1976) 161-163.

19. Gruener, N., "Ontogenetic Development of NADH Dependent Methemoglobin Reductase in Erythrocytes of Different Species," Comparative Biochem. & Physiol. J. Toxicol. Environ. Health., 1(5):787-791, 1976.

20. Shechter, M. & Gruener, N., " An Evaluation of the Ion Selective Electrode for Determination of Nitrate in Highly Mineralized Drinking Water," J. Am. Water Works. Assn. 68:363, 1976.